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<b>(21) International Application Number:</b> PCT/US92/00992 <b>(22) International Filing Date:</b> 5 February 1992 (05.02.92)  <b>(30) Priority data:</b> 652,427 6 February 1991 (06.02.91) US 827,269 3 February 1992 (03.02.92) US 827,270 3 February 1992 (03.02.92) US  <b>(71) Applicant:</b> IGEN, INC. [US/US]; 1530 East Jefferson Street, Rockville, MD 20852 (US).  <b>(72) Inventors:</b> LELAND, John, K. ; 14002 Chestnut Court, Laurel, MD 20707 (US). SHAH, Haresh, P. ; 21 Norwich Court, Gaithersburg, MD 20878 (US). KENTEN, John, H. ; 1921 Windjammer Way, Gaithersburg, MD 20879 (US). GOODMAN, Jack, E. ; 3011 Third Street North, Arlington, VA 22201 (US). LOWKE, George, E. ; 21706 Mobley Farm Drive, Laytonsville, MD 20882 (US). BLACKBURN, Gary, F. ; 23627 Rolling Fork Way, Gaithersburg, MD 20882 (US). MASSEY, Richard, J. ; 5 Valerian Court, Rockville, MD 20852 (US).		<b>(74) Agents:</b> EVANS, Barry et al.; Curtis, Morris & Safford, 530 Fifth Avenue, New York, NY 10036 (US).  <b>(81) Designated States:</b> AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, B. (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC (European patent), MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, RU, SD, SE, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent).  <b>Published</b> <i>With international search report.</i>  <b>BEST AVAILABLE COPY</b>
<b>(54) Title:</b> METHODS AND APPARATUS FOR IMPROVED LUMINESCENCE ASSAYS  <b>(57) Abstract</b>  The invention relates to methods, apparatus, reagents, and kits for performing a binding assay for an analyte of interest present in a sample based upon electrochemiluminescence at an electrode of interest. In the method, reagents and kits particles can be employed; for instance, for setting upon the electrode surface by gravity, centrifugation or magnetic attraction. The apparatus can include a magnet for generating a magnetic field in a region proximate the electrode.		

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Methods and Apparatus for Improved  
Luminescence Assays

Cross Reference To Related Applications

This application is a continuation-in-part of  
5 copending Massey et al. application Ser.  
No. 07/652,427, filed February 6, 1991, which in turn  
is a continuation-in-part of application Ser. No.  
266,882, filed November 3, 1988, now abandoned,  
entitled Electrochemiluminescent Assays; this  
10 application is also a continuation-in-part of  
application Ser. No. 539,389, filed June 18, 1990,  
which is a continuation of application Serial No.  
266,882. Reference is also made to the concurrently  
filed application of Massey et al., Serial No.  
15 07/827,269 entitled "Method and Apparatus For  
Magnetic Microparticulate Based Luminescence Assay  
Including Plurality of Magnets" (CMS docket 370068-  
3401), which is also a continuation-in-part of  
application Serial No. 07/652,427. All of the above-  
20 referenced applications are hereby incorporated herein  
by reference.

Field of The Invention

This application relates generally to methods  
and apparatus for conducting binding assays, more  
25 particularly to those which measure the presence of an  
analyte of interest by measuring luminescence emitted  
by one or more labeled components of the assay system.  
More specifically, the invention relates to precise,  
reproducible, accurate homogeneous or heterogeneous  
30 specific binding assays of improved sensitivity in  
which the luminescent component is concentrated in the  
assay composition and collected on the detection system  
before being caused to electrochemiluminescence.

Background of The Invention

35 Numerous methods and systems have been  
developed for the detection and quantitation of

analytes of interest in biochemical and biological substances. Methods and systems which are capable of measuring trace amounts of microorganisms, pharmaceuticals, hormones, viruses, antibodies, nucleic acids and other proteins are of great value to researchers and clinicians.

A very substantial body of art has been developed based upon the well known binding reactions, e.g., antigen-antibody reactions, nucleic acid hybridization techniques, and protein-ligand systems. The high degree of specificity in many biochemical and biological binding systems has led to many assay methods and systems of value in research and diagnostics. Typically, the existence of an analyte of interest is indicated by the presence or absence of an observable "label" attached to one or more of the binding materials. Of particular interest are labels which can be made to luminesce through photochemical, chemical, and electrochemical means.

"Photoluminescence" is the process whereby a material is induced to luminesce when it absorbs electromagnetic radiation. Fluorescence and phosphorescence are types of photoluminescence. "Chemiluminescent" processes entail the creation of luminescent species by chemical transfer of energy. "Electrochemiluminescence" entails creation of luminescent species electrochemically.

Chemiluminescent assay techniques where a sample containing an analyte of interest is mixed with a reactant labeled with a chemiluminescent label have been developed. The reactive mixture is incubated and some portion of the labeled reactant binds to the analyte. After incubation, the bound and unbound fractions of the mixture are separated and the concentration of the label in either or both fractions can be determined by chemiluminescent techniques. The level of chemiluminescence determined in one or both



fractions indicates the amount of analyte of interest in the biological sample.

Electrochemiluminescent (ECL) assay techniques are an improvement on chemiluminescent techniques. They provide a sensitive and precise measurement of the presence and concentration of an analyte of interest. In such techniques, the incubated sample is exposed to a voltammetric working electrode in order to trigger luminescence. In the proper chemical environment, such electrochemiluminescence is triggered by a voltage impressed on the working electrode at a particular time and in a particular manner. The light produced by the label is measured and indicates the presence or quantity of the analyte. For a fuller description of such ECL techniques, reference is made to PCT published application US85/01253 (W086/02734), PCT published application number US87/00987, and PCT published application U.S. 88/03947. The disclosures of the aforesaid applications are incorporated by reference.

It is desirable to carry out electrochemiluminescent assays without the need for a separation step during the assay procedure and to maximize the signal modulation at different concentrations of analyte so that precise and sensitive measurements can be made. Among prior art methods for nonseparation assays are those which employ microparticulate matter suspended in the assay sample to bind one or more of the binding components of the assay.

U.S. Patent No. 4,305,925 relates to the detection and determination of clinically relevant proteins and peptides by means of nephelometric and turbidimetric methods. The methods disclosed involve binding the antigen or antibody to latex particles which perform the function of light scattering or adsorption.

U.S. Patent No. 4,480,042 relates to techniques employing particle reagents consisting of shell-core particles. The shell contains functional groups to which compounds of biological interest can be covalently bonded, and the high refractive index of the core results in high sensitivity to light scattering measurements. The technique is based upon agglutination reactions which result from the reaction of bivalent antibodies with multivalent antigens of interest to produce aggregates which can be detected and/or measured in various ways.

U.S. Patent No. 4,419,453 likewise relates to the use of colored latex agglutination test methods useful for detecting the presence of immunochemicals such as antibodies and immunogens.

Based upon this prior art, it would not have appeared possible to use microparticulate matter in assays wherein a luminescent phenomenon is measured. One would expect that the luminescence from free chemiluminescent or electrochemiluminescent moieties would be absorbed, scattered, or otherwise suffer interference from the microparticulate matter.

Contrary to that expectation, U.S. Application Serial No. 539,389 (PCT published application U.S. 89/04919) teaches sensitive, specific binding assay methods based on a luminescent phenomenon wherein inert microparticulate matter is specifically bound to one of the binding reactants of the assay system. The assays may be performed in a heterogeneous (one or more separation steps) assay format and may be used most advantageously in a homogeneous (nonseparation) assay format.

U.S. 89/04919 relates to a composition for an assay based upon a binding reaction for the measurement of luminescent phenomenon, which composition includes a plurality of suspended particles having a surface

capable of binding to a component of the assay mixture. In another aspect, it is directed to a system for detecting or quantitating an analyte of interest in a sample, which system is capable of conducting the assay methods using the assay compositions of the inventions. The system includes means for inducing the label compound in the assay medium to luminesce, and means for measuring the luminescence to detect the presence of the analyte of interest in the sample.

It was found that the binding of that component of the assay system to which an electrochemiluminescent moiety has been linked, to suspended microparticulate matter, greatly modulates the intensity of the luminescent signal generated by the electrochemiluminescent moiety linked to that component, thereby providing a means of monitoring the specific binding reaction of the assay system. Even more surprisingly, the suspended particles were found to have little or no effect on the intensity of the luminescent signal generated by the electrochemiluminescent moiety linked to the component of the system which remains unbound to the suspended microparticulate matter.

Thus, U.S. 89/04919 is directed to methods for the detection of an analyte of interest in a sample, which method includes the steps of (1) forming a composition comprising (a) a sample suspected of containing an analyte of interest, (b) an assay-performance-substance selected from the group consisting of (i) analyte of interest or analog of the analyte of interest, (ii) a binding partner of the analyte of interest or its said analog, and (iii) a reactive component capable of binding with (i) or (ii), wherein one of said substances is linked to a label compound having a chemical moiety capable of being induced to luminesce, and (c) a plurality of suspended

particles capable of specifically binding with the analyte and/or a substance defined in (b)(i), (ii), or (iii); (2) incubating the composition to form a complex which includes a particle and said label compound; (3) inducing the label compound to luminesce; and (4) measuring the luminescence emitted by the composition to detect the presence of the analyte of interest in the sample. Those same methods may be used to quantify the amount of analyte in a sample by comparing the luminescence of the assay composition to the luminescence of a composition containing a known amount of analyte.

Analogs of the analyte of interest, which may be natural or synthetic, are compounds which have binding properties comparable to the analyte, but include compounds of higher or lower binding capability as well. Binding partners suitable for use in the present invention are well-known. Examples are antibodies, enzymes, nucleic acids, lectins, cofactors and receptors. The reactive components capable of binding with the analyte or its analog and/or with a binding partner thereof may be a second antibody or a protein such as Protein A or Protein G or may be avidin or biotin or another component known in the art to enter into binding reactions.

Advantageously, the luminescence arises from electrochemiluminescence (ECL) induced by exposing the label compound, whether bound or unbound to specific binding partners, to a voltammetric working electrode. The ECL reactive mixture is controllably triggered to emit light by a voltage impressed on the working electrode at a particular time and in a particular manner to generate light. Although the emission of visible light is an advantageous feature the composition or system may emit other types of electromagnetic radiation, such as infrared or ultraviolet

light, X-rays, microwaves, etc. Use of the terms "electrochemiluminescence," "electrochemiluminescent," "luminescence," "luminescent," and "luminesce" includes the emission of light and other forms of  
5 electromagnetic radiation.

The methods taught in U.S. 89/04919 permit the detection and quantitation of extremely small quantities of analytes in a variety of assays performed in research and clinical settings. The demands of  
10 researchers and clinicians makes it imperative, however, to lower the detection limits of assays performed by these methods to increase the sensitivities of those assays and to increase the speed at which they can be performed.

15 Various methods are known in the art for increasing the signal from labeled species by concentrating them before subjecting them to a measurement step. In U.S. Patent No. 4,652,333, for example, particles labeled with fluorescent,  
20 phosphorescent or atomic fluorescent labels are concentrated by microfiltration before a measurement step is performed.

It is also known in the art to concentrate labeled immunochemical species prior to a measurement  
25 step, by, e.g., drawing magnetically responsive labeled particles to the surface of a measurement vessel. In U.S. Patents No. 4,731,337, 4,777,145, and 4,115,535, for example, such particles are drawn to the vessel wall and then are irradiated to excite a fluorophoric  
30 emission of light.

In U.S. Patent No. 4,945,045, particles are concentrated on a magnetic electrode. An  
electrochemical reaction takes place at the electrode facilitated by a labeled chemical mediator. The  
35 immunochemical binding reaction alters the efficiency

of the mediator resulting in a modulated signal when binding takes place.

These prior art methods are not relevant to the surface selective excitation processes of the invention. While not being bound by any particular mechanistic explanation of surface excitation, e.g., electrochemiluminescence, it is believed that the label on the solid-phase complex must be oxidized at the electrode. This requires that an electron move from the label to the electrode. It is believed that the electron makes this "jump" by a phenomenon known as tunneling in which the electron passes through space (a region where its potential energy is very high, e.g., the solution) without having to go "over" the potential energy barrier. It can tunnel through the energy barrier, and thus, move from one molecule to another or from one molecule to an electrode without additional energy input. However, this tunneling phenomenon can only operate for very short distances. The probability of the tunneling phenomenon falls off exponentially as the distance between the two species increases. The probability of the tunneling phenomenon occurring between two species is fairly high if the distance is less than 25 Angstroms (2.5 nm) but is fairly low if the distance is greater. The distance of 25 Å is a rule-of-thumb used by those skilled in the art but is not an absolute limitation.

Accordingly, only those ECL labels with 25 Å of the surface of the electrode can be expected to participate in the ECL process. The area of the particle which is within 25 Å of the surface of an electrode is typically extremely small.

Accordingly, one would not expect that ECL from a particle surface would be measurable to any significant degree. Moreover, the light which is produced by the ECL process must pass through the

particle to get to the photomultiplier. Since the particles are essentially opaque (a concentrated suspension of them is black) one would not expect that, even if significant amounts of light could be produced by ECL, that the light could pass through the particle and be measured by the photomultiplier.

#### Objects of The Invention

It is therefore a primary object of this invention to provide homogeneous (non-separation) and heterogeneous (separation) methods, reagents and apparatus, for the conduct of binding assays.

It is a further object of this invention to provide non-separation, specific bonding assays, reagents and apparatus, based upon the measurement of electrochemiluminescence emitted from an assay composition containing microparticulate matter.

It is a further and related object to provide such assays, reagents and apparatus having improved sensitivity, faster assay time, greater specificity, lower detection limits and greater precision than has heretofore been achieved.

#### Description of the Invention

##### Definition of Terms

The term "ECL moiety," "metal-containing ECL moiety" "label," "label compound," and "label substance," are used interchangeably. It is within the scope of the invention for the species termed "ECL moiety," "metal-containing ECL moiety," "organo-metallic," "metal chelate," "transition metal chelate" "rare earth metal chelate," "label compound," "label substance" and "label" to be linked to molecules such as an analyte or an analog thereof, a binding partner of the analyte or an analog thereof, and further binding partners of such aforementioned binding

partner, or a reactive component capable of binding with the analyte, an analog thereof or a binding partner as mentioned above. The above-mentioned species can also be linked to a combination of one or more binding partners and/or one or more reactive components. Additionally, the aforementioned species can also be linked to an analyte or its analog bound to a binding partner, a reactive component, or a combination of one or more binding partners and/or one or more reactive components. It is also within the scope of the invention for a plurality of the aforementioned species to be bound directly, or through other molecules as discussed above, to an analyte or its analog. For purposes of brevity, these ligands are referred to as an assay-performance-substance.

The terms detection and quantitation are referred to as "measurement", it being understood that quantitation may require preparation of reference compositions and calibrations.

The terms collection and concentration of complex may be used interchangeably to describe the concentration of complex within the assay composition and the collection of complex at, e.g., an electrode surface.

#### Brief Description of the Drawings

Fig. 1 is a schematic drawing of a cell for performing the microparticulate-based nonseparation and separation assays of the invention.

Fig. 2 is a simplified diagram of a voltage control apparatus for use with the cell of Fig. 1.

Fig. 3 is a schematic representation of a direct incorporation PCR format using electrochemiluminescent labeled oligonucleotides and biotin electrochemiluminescent labeled oligonucleotides as primers.



Fig. 4 is a schematic representation of a normal PCR format using a biotinylated primer to allow the generation of biotinylated PCR PRODUCT.

5 Fig. 5 is a schematic representation of an asymmetric PRC assay format generating single-stranded biotinylated DNA for later hybridization to electrochemiluminescent labeled oligonucleotides.

10 Fig. 6 is a graph showing specificity studies of the direct incorporation of electrochemiluminescent labeled oligonucleotides into biotinylated PCR products.

15 Fig. 7 is a standard curve of directly incorporated electrochemiluminescent label and biotinylated oligonucleotides into HPV16 PCR products.

Fig. 8 is a graph showing a point mutation assay for the Ha-ras oncogene.

20 Fig. 9 is a graph showing an evaluation of the specificity of electrochemiluminescent labeled probes using  $P^{32}$  electrochemiluminescent labeled probes for the Aa-ras oncogene.

25 Fig. 10 is a graph showing the determination of the relative value of electrochemiluminescent label in  $P^{32}$  electrochemiluminescent label for the determination of point mutations in the Ha-ras oncogene.

Fig. 11 is a standard curve of the rapid "no wash" hybridization assay for HPV18.

30 Fig. 12 is a schematic representation of an assay cell used to conduct assays relying upon gravitational force to cause the complex to settle.

Fig. 13 is a graph showing the distance the complex settles as a function of time under influence of gravity; namely, a sedimentation rate of Dynal Particles ( $y = -0.28 + 0.48x$ ; speed = 0.5mm per min).

35 Fig. 14 is a graph showing the intensity of electrochemiluminescence as a function of time in

gravity cells having different heights of assay composition over the electrode, i.e., a comparison of the intensity-time relationship for two gasket thicknesses, wherein the values represented by the open circles are for a 0.015" gasket and the values represented by the darkened circles are for a 0.075" gasket.

Fig. 15 is a graph showing the intensity of electrochemiluminescence in gravity cells having different heights of assay composition over the electrode surface as measured in an assay for the measurement of alpha fetal protein, i.e., a comparison of cells for AFP Assay, wherein the values represented by the open circles are for a 0.015" gasket and the values represented by the darkened circles are for a 0.075" gasket.

Fig. 16 is a schematic representation of a sedimentation assay cell which employs an electromagnet to cause the complex to settle on the electrode surface.

Fig. 17 is a graph showing the relative rates of settling of microparticulate complex under the influence of a magnetic field and of gravity, respectively, i.e., a comparison of microparticulate settling times between magnetic field induced settling and gravity settling, wherein values for the magnetic field settling are represented by open circles and the values for gravity settling are represented by darkened circles.

Fig. 18 is a schematic representation of a collection cell including a permanent magnet.

Fig. 19 is a graph showing the increase in ECL intensity as a function of time in assays conducted with the cell of Fig. 18, i.e., the effect of collection time on ECL intensity.

Fig. 20 is a schematic representation of the lines of force in the vicinity of the electrode surface as a function of the orientation of the magnet beneath the electrode surface.

5 Fig. 21 is a schematic representation of a rotary flow cell wherein the complexes are deposited upon the surface of the electrode by centrifugation; the centrifugal method and apparatus of the invention for capturing particles; the centrifugal flow cell of  
10 the invention.

Fig. 22 is a schematic representation of an evanescent-wave fluorescence detection.

Figs. 23 and 24 show a cell and plurality of magnets for performing the magnetic microparticulate-based separation or non-separation assay method of the  
15 invention; the plurality of magnets of the magnet system imposes the field lines which are largely parallel to the plane of the electrode surface.

Fig. 25 is a schematic drawing of a cell for  
20 performing the microparticulate-based non-separation and separation assays of the invention; the cell employs a working electrode and plurality of magnets as in Figs. 23 and 24.

#### Brief Description of the Invention

25 In its broadest embodiment, the invention is in a method for performing a binding assay for an analyte of interest present in a sample. The steps include:

- (a) forming a composition containing
  - 30 (i) said sample
  - (ii) an assay-performance-substance which contains a component linked to a label compound capable of being induced to luminesce, and

## 13.1

- (iii) a plurality of particles capable of specifically binding with the analyte and/or said assay-performance-substance;
- 5 (b) incubating said composition to form a complex which includes a particle and said label compound;
- (c) collecting said complex in a measurement zone;
- 10 (d) inducing the label compound in said complex to luminesce by surface selective excitation; and

(e) measuring the emitted luminescence to measure the presence of the analyte of interest in the sample.

The complex may be collected on, e.g., an electrode surface where it is excited and induced to electrochemiluminesce, as by impressing a voltage on the electrode, or, it may be collected on a surface and be thereafter induced to fluoresce by surface excitation as described below. Total-internal-reflection-fluorescence (TIRF) has been described as a surface sensitive technique for exciting and detecting fluorophoric labels and total-internal-reflection has been used with RAMAN and infra-red absorption as another surface-sensitive technique for measuring the presence of a label. Surface plasmon resonance is an optical technique which may be used according to methods of the invention to measure labels on surfaces. The invention is thus directed to methods for exciting luminescence by surface excitation techniques.

While the invention is preferably carried out by collecting the complex in a measurement zone, i.e., on a surface at which it can be caused to luminesce, the invention also embraces methods wherein the complex is collected outside a measurement zone and thereafter means are brought to that zone or other steps taken to induce and measure luminescence.

The collection of the complex may be carried out by several different methods, including gravity settling, filtration, centrifugation and magnetic attraction of magnetically responsive particles which form part of the complex. The several embodiments are described in further detail below.

Assays based upon the measurement of electrochemiluminescence at an electrode surface are advantageously carried out using the forces of gravity by

- 5 (a) forming a composition containing  
(i) said sample  
(ii) an assay-performance-substance  
which contains a component linked  
to a label compound capable of  
being induced to  
electrochemiluminesce, and  
(iii) a plurality of suspended particles  
10 having a density greater than the  
balance of said composition and  
being capable of specifically  
binding with the analyte and or  
said assay-performance-substance;
- 15 (b) incubating said composition to form a  
complex which includes a particle and said label  
compound;
- (c) introducing said composition into an  
assay cell;
- 20 (d) collecting said complex at the surface  
of an electrode located below at least a  
substantial portion of the volume of said assay  
cell by permitting said composition to reside in  
said cell for a time sufficient to permit the  
particles to settle upon said electrode surface by  
25 the force of gravity;
- (e) inducing the label compound in said  
collected complex to luminescence by imposing a  
voltage on said electrode; and
- 30 (f) measuring the emitted luminescence at  
the electrode surface to measure the presence of  
the analyte of interest in the sample.

While batch assays can be performed,  
continuous or semi-continuous assays can be performed  
in flow cells. In a flow cell, the solid-phase remains  
35 in the measurement cell while the solution flows  
through and exits the cell. If the solid-phase (e.g.,

particles) are more dense than water, i.e., have a density greater than that of water, (more than 1.0 g/mL) the force of gravity upon the particles causes them to fall to the bottom of the cell. The cell can be constructed such that the particles settle to the bottom as the fluid flows through the cell or the cell can be constructed such that the majority of the sample is contained in the cell in a columnar compartment above the working electrode of an ECL system.

Sufficient dwell time in the cell must be provided to permit the particles to settle on the surface of the electrode before inducing ECL.

In another embodiment of the invention, the assay composition containing suspended particles having a density greater than the balance of the assay composition may be subjected to centrifugation in order to remove the particles to a measurement zone where they are subsequently brought into contact with, e.g., an electrode to induce electrochemiluminescence or brought directly into contact with an electrode in the centrifugation step.

In this embodiment, the measurement cell is provided with means to rapidly rotate the sample and sample enclosure. Centrifugal force causes the particles in the sample to move outward from the axis of rotation of the sample enclosure and to collect on the outer surface of the sample enclosure. The outer surfaces of such sample enclosure may constitute the working electrode of an ECL measurement system.

In a third embodiment, the particles may be removed by filtration from the assay composition. In this embodiment the particles need not have a density greater than the balance of the assay composition. The invention, the particles are separated from the solution and concentrated by drawing the solution through a filter, e.g. pumping and collecting the

particles on the surface of the filter. This surface of the filter is, for example, coated with a thin metal film which can serve as the working electrode in an ECL detection system.

5           In a preferred embodiment, the suspended particles are magnetically responsive, e.g. they may be paramagnetic or ferromagnetic, and are collected in a measurement zone or, preferably, directly at the surface of an electrode, by imposition of a magnetic  
10   field on the particles. The measurement cell is equipped with a magnet. The magnetic field of the magnet applies a force on the particles as they reside in a batch cell or as they flow through a flow cell, causing them to separate from the bulk of the solution  
15   onto the surface of the cell which is in closest proximity to the magnet. If the magnet is placed in a proper orientation and in close proximity to the working electrode of an ECL detection system the particles will concentrate on the surface of the  
20   working electrode.

          Several different heterogeneous and homogeneous formats for binding assays can be implemented using the methods described above to collect and concentrate the complex on the surface of  
25   an electrode. In a heterogeneous binding assay the complex is separated from the composition before measuring luminescence from the label. In homogeneous assays, no separation of the bound (to the solid phase) and unbound labeled reagents is made.

30           In a heterogenous assay, when the complex is concentrated on the surface of the working electrode, the measured signal from the label is much greater than it would be in the absence of a collection step. The signal from the uncomplexed labeled reagents, in  
35   contrast, is not changed. Hence, despite the presence of the uncomplexed labeled reagents in the measurement



cell, the signal from the collected complex is stronger than in an assay without collection of complex. The detection limit for the binding assay is, much improved as a result of the collection procedure.

5 In a preferred embodiment of the invention, an *in-situ* separation step is included in the homogeneous binding assay procedure. After the assay composition, i.e., sample, assay performance substance and particles have been pumped into the measurement  
10 cell and the complex captured upon the working electrode, a second fluid is pumped through the cell which is free of label or labeled reagents, thereby performing an *in-situ* wash or separation of the complex from unbound components of the assay composition. This  
15 assay procedure is technically a heterogeneous binding assay. However, the ability to perform the separation inside the measurement cell is advantageous in that it does not require additional separation apparatus and the procedure is generally much faster than external  
20 separation methods.

Heterogeneous binding assays are conducted using the invention by mixing the components of the assay composition and allowing them to react for a predetermined length of time. The assay composition is  
25 then subjected to a separation step wherein the solution is separated from the particles. Electrochemiluminescence is then measured from either the complex or the solution. Measuring the ECL from the complex after a concentration step permits  
30 measurement of analyte with better accuracy and with a lower detection limit than is possible without concentration.

#### Detailed Description of the Invention

35 The invention, as well as other objects and features thereof, will be understood more clearly and

fully from the following description of certain preferred embodiments.

The invention is broadly applicable to analytes of interest which are capable of entering into binding reactions. These reactions include, e.g., antigen-antibody, ligand receptor, DNA and RNA interactions, and other known reactions. The invention relates to different methods and assays for qualitatively and quantitatively detecting the presence of such analytes of interest in a multicomponent sample.

#### The Samples

The sample which may contain the analyte of interest, which may be in solid, emulsion, suspension, liquid, or gas form, may be derived from, for example, cells and cell-derived products, water, food, blood, serum, hair, sweat, urine, feces, tissue, saliva, oils, organic solvents or air. The sample may further comprise, for example, water, acetonitrile, dimethyl sulfoxide, dimethyl formamide, n-methyl-pyrrolidone or alcohols or mixtures thereof.

#### 25 The Analytes

Typical analytes of interest are a whole cell or surface antigen, subcellular particle, virus, prion, viroid, antibody, antigen, hapten, fatty acid, nucleic acid, protein, lipoprotein, polysaccharide, lipopolysaccharide, glycoprotein, peptide, polypeptide, cellular metabolite, hormone, pharmacological agent, synthetic organic molecule, organometallic molecule, tranquilizer, barbiturate, alkaloid, steroid, vitamin, amino acid, sugar, lectin, recombinant or derived protein, biotin, avidin, streptavidin, or inorganic

molecule present in the sample. Typically, the analyte of interest is present at a concentration of  $10^{-3}$  molar or less, for example, as low as  $10^{-12}$  molar or lower.

5     Assay-Performance-Substance

          The assay-performance-substance which is combined with the sample containing the analyte of interest contains at least one substance selected from the group consisting of (i) added analyte of interest or its analog, as defined above, (ii) a binding partner of the analyte of interest or its said analog, and (iii) a reactive component, as defined above, capable of binding with (i) or (ii), wherein one of said substances is linked to a compound or moiety, e.g. an ECL moiety capable of being induced to luminesce. The labeled substance may be a whole cell or surface antigen, a subcellular particle, virus, prion, viroid, antibody, antigen, hapten, lipid, fatty acid, nucleic acid, polysaccharide, protein, lipoprotein, lipopolysaccharide, glycoprotein, peptide, polypeptide, cellular metabolite, hormone, pharmacological agent, tranquilizer, barbiturate, alkaloid, steroid, vitamin, amino acid, sugar, nonbiological polymer (preferably soluble), lectin, recombinant or derived protein, synthetic organic molecule, organometallic molecule, inorganic molecule, biotin, avidin or streptavidin. In one embodiment, the reagent is an electrochemiluminescent moiety conjugated to an antibody, antigen, nucleic acid, hapten, small nucleotide sequence, oligomer, ligand, enzyme, or biotin, avidin, streptavidin, Protein A, Protein G, or complexes thereof, or other secondary binding partner capable of binding to a primary binding partner through protein interactions.

Analogs of the analyte of interest, which can be natural or synthetic, are typically compounds which have binding properties comparable to the analyte, but can also be compounds of higher or lower binding capability. The reactive component capable of binding with the analyte or its analog, and/or with a binding partner thereof, and through which the ECL moiety can be linked to the analyte, is suitably a second antibody or a protein such as Protein A or Protein G, or avidin or biotin or another component known in the art to enter into binding reactions.

#### The Labels

Advantageously, the ECL moieties are metal chelates. The metal of that chelate is suitably any metal such that the metal chelate will luminesce under the electrochemical conditions which are imposed on the reaction system in question. The metal of such metal chelates is, for instance, a transition metal (such as a d-block transition metal) or a rare earth metal. The metal is preferably ruthenium, osmium, rhenium, iridium, rhodium, platinum, indium, palladium, molybdenum, technetium, copper, chromium or tungsten. Especially preferred are ruthenium and osmium.

The ligands which are linked to the metal in such chelates are usually heterocyclic or organic in nature, and play a role in determining whether or not the metal chelate is soluble in an aqueous environment or in an organic or other nonaqueous environment. The ligands can be polydentate, and can be substituted. Polydentate ligands include aromatic and aliphatic ligands. Suitable aromatic polydentate ligands include aromatic heterocyclic ligands. Preferred aromatic heterocyclic ligands are nitrogen-containing, such as, for example, bipyridyl, bipyrazyl, terpyridyl, and

phenanthrolyl. Suitable substituents include for example, alkyl, substituted alkyl, aryl, substituted aryl, aralkyl, substituted aralkyl, carboxylate, carboxaldehyde, carboxamide, cyano, amino, hydroxy, imino, hydroxycarbonyl, aminocarbonyl, amidine, guanidinium, ureide, sulfur-containing groups, phosphorus containing groups, and the carboxylate ester of N-hydroxysuccinimide. The chelate may have one or more monodentate ligands, a wide variety of which are known to the art. Suitable monodentate ligands include, for example, carbon monoxide, cyanides, isocyanides, halides, and aliphatic, aromatic and heterocyclic phosphines, amines, stilbenes, and arsines.

Examples of suitable chelates are bis [(4,4'-carbomethoxy)-2,2'-bipyridine] 2-[3-(4-methyl-2,2'-bipyridine-4-yl)propyl]-1,3-dioxolane ruthenium (II); bis (2,2'-bipyridine) [4-(butan-1-yl)-4'-methyl-2,2'-bipyridine] ruthenium (II); bis (2,2'-bipyridine) [4-(4'-methyl-2,2'-bipyridine-4'-yl)-butyric acid] ruthenium (II); tris (2,2'-bipyridine) ruthenium (II); (2,2'-bipyridine) [bis-bis(1,2-diphenylphosphino)ethylene] 2-[3-(4-methyl-2,2'-bipyridine-4'-yl)propyl]-1,3-dioxolane osmium (II); bis (2,2'-bipyridine) [4-(4'-methyl-2,2'-bipyridine)-butylamine] ruthenium (II); bis (2,2'-bipyridine) [1-bromo-4(4'-methyl-2,2'-bipyridine-4-yl)butane] ruthenium (II); bis (2,2'-bipyridine)maleimidoheptanoic acid, 4-methyl-2,2'-bipyridine-4'-butylamide ruthenium (II). Other ECL moieties are described in PCT published application US87/00987 and PCT published application 88/0394, incorporated herein by reference.

The function of the ECL moieties is to emit electromagnetic radiation as a result of introduction into the reaction system of electrochemical energy. In order to do this, they must be capable of being

stimulated to an excited energy state and also capable of emitting electromagnetic radiation, such as a photon of light, upon descending from that excited state.

While not wishing to be bound by theoretical analysis of the mechanism of the ECL moiety's participation in the electrochemiluminescent reaction, we believe that it is oxidized by the introduction of electrochemical energy into the reaction system and then, through interaction with a reductant present in the system, is converted to the excited state. This state is relatively unstable, and the metal chelate quickly descends to a more stable state. In so doing, the chelate gives off electromagnetic radiation, such as a photon of light, which is detectable.

The amount of metal chelate or other metal-containing ECL moiety incorporated in accordance with the invention will vary from system to system. Generally, the amount of such moiety utilized is that amount which is effective to result in the emission of a detectable, and if desired, quantifiable, emission of electromagnetic energy, from the aforementioned composition or system. The detection and/or quantitation of an analyte of interest is typically made from a comparison of the luminescence from a sample containing an analyte of interest and an ECL moiety to the luminescence emitted by a calibration standard developed with known amounts of the analyte of interest and ECL moiety. This assumes a homogeneous format. In the heterogeneous mode, a separation as discussed previously is carried out prior to ECL analysis.

As can be appreciated by one of ordinary skill in the art, the identity and amount of the metal-containing ECL moiety will vary from one system to another, depending upon prevailing conditions. The appropriate metal-containing ECL moiety, and

sufficient amount thereof to obtain the desired result, can be determined empirically by those of ordinary skill in the art, once equipped with the teachings herein, without undue experimentation.

5

### The Particles

The particles advantageously comprise micro-particulate matter having a diameter of 0.001 to 200  $\mu\text{m}$ , such as 0.05  $\mu\text{m}$  to 200  $\mu\text{m}$ , preferably 0.1  $\mu\text{m}$  to 100  $\mu\text{m}$ , most preferably 0.5  $\mu\text{m}$  to 10  $\mu\text{m}$ , and a surface component capable of binding to the analyte and/or one or more of the other substances defined above. For example, the microparticulate matter may be crosslinked starch, dextrans, cellulose, proteins, organic polymers, styrene copolymer such as styrene/butadiene copolymer, acrylonitrile/butadiene/styrene copolymer, vinylacetyl acrylate copolymer, or vinyl chloride/acrylate copolymer, inert inorganic particles, chromium dioxide, oxides of iron, silica, silica mixtures, and proteinaceous matter, or mixtures thereof. Desirably, the particles are suspended in the ECL system. The particles can be or can include magnetically responsive particles.

25

### Assay Media

In order to operate a system in which an electrode introduces electrochemical energy, it is necessary to provide an electrolyte in which the electrode is immersed and which contains the ECL moiety. The electrolyte is a phase through which charge is carried by ions. Generally, the electrolyte is in the liquid phase, and is a solution of one or more salts or other species in water, an organic liquid

30

35

or mixture of organic liquids, or a mixture of water and one or more organic liquids. However, other forms of electrolyte are also useful in certain embodiments of the invention. For example, the electrolyte may be  
5 a dispersion of one or more substances in a fluid -- e.g., a liquid, a vapor, or a supercritical fluid -- or may be a solution of one or more substances in a solid, a vapor or supercritical fluid.

The electrolyte is suitably a solution of a  
10 salt in water. The salt can be a sodium salt or a potassium salt preferably, but incorporation of other cations is also suitable in certain embodiments, as long as the cation does not interfere with the electrochemiluminescent interaction sequence. The  
15 salt's anion may be a phosphate, for example, but the use of other anions is also permissible in certain embodiments of the invention --once again, as long as the selected anion does not interfere with the electrochemiluminescent interaction sequence.

20 The composition may also be nonaqueous. While supercritical fluids can in certain instances be employed advantageously, it is more typical to utilize an electrolyte comprising an organic liquid in a nonaqueous composition. Like an aqueous electrolyte,  
25 the nonaqueous electrolyte is also a phase through which charge is carried by ions. Normally, this means that a salt is dissolved in the organic liquid medium. Examples of suitable organic liquids are acetonitrile, dimethylsulfoxide (DMSO), dimethylformamide (DMF),  
30 methanol, ethanol, and mixtures of two or more of the foregoing. Illustratively, tetraalkylammonium salts, such as tetrabutylammonium tetrafluoroborate, which are soluble in organic liquids can be used with them to form nonaqueous electrolytes.

35 The electrolyte is, in certain embodiments of the invention, a buffered system. Phosphate buffers



are often advantageous. Examples are an aqueous solution of sodium phosphate/sodium chloride, and an aqueous solution of sodium phosphate/sodium fluoride.

5     Other Assay Components

As described PCT published application U.S. 89/04859, entitled Electrochemiluminescent Reaction Utilizing Amine-Derived Reductant, the disclosure of which is incorporated by reference, it is desirable to include a reductant, typically an amine or amine moiety (of a larger molecule) which can be oxidized and spontaneously decomposed to convert it into a highly reducing species. It is believed that the amine or amine moiety is also oxidized by electrochemical energy introduced into the reaction system. The amine or amine moiety loses one electron, and then deprotonates, or rearranges itself, into a strong reducing agent. This agent interacts with the oxidized metal-containing ECL moiety and causes it to assume the excited state discussed above. In order to carry out its role, the amine or amine moiety preferably has a carbon-centered radical with an electron which can be donated from such carbon, and an alpha carbon which can then act as a proton donor during deprotonation in order to form the reductant. The amine-derived reductant provides the necessary stimulus for converting the metal-containing ECL moiety to its excited state, from which detectable electromagnetic radiation is emitted.

30             A wide range of amines and corresponding amine moieties can be utilized in practicing the present invention. Generally, the amine or amine moiety is chosen to suit the pH of the system which is to be electrochemiluminescently analyzed. Another relevant factor is that the amine or amine moiety should be compatible with the environment in which it

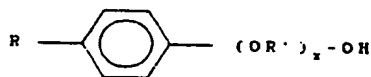
must function during analysis, i.e., compatible with an aqueous or nonaqueous environment, as the case may be. Yet another consideration is that the amine or amine moiety selected should form an amine-derived reductant under prevailing conditions which is strong enough to reduce the oxidized metal-containing ECL moiety in the system.

Amines (and corresponding moieties derived therefrom) which are advantageously utilized in the present invention are aliphatic amines, such as primary, secondary and tertiary alkyl amines, the alkyl groups of each having from one to three carbon atoms, as well as substituted aliphatic amines. Tripropyl amine is an especially preferred amine as it leads to, comparatively speaking, a particularly high-intensity emission of electromagnetic radiation, which enhances the sensitivity and accuracy of detection and quantitation with embodiments in which it is used. Also suitable are diamines, such as hydrazine, and polyamines, such as poly(ethyleneimine). Examples of other amines suitable for practicing the invention are triethanol amine, triethyl amine, 1,4-diazabicyclo-(2.2.2)-octane, 1-piperidine ethanol, 1,4-piperazine-bis-(ethane-sulfonic acid), tri-isopropyl amine and poly(ethyleneimine).

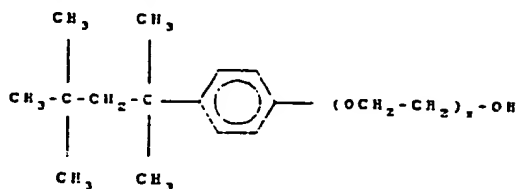
Typically, the metal-containing ECL moiety utilized in the present invention is the reaction-limiting constituent. Accordingly, it is also typical that the amine or amine moiety is provided in a stoichiometric excess with respect thereto. Illustratively, the amine or amine moiety is employed in a concentration of 50-150 mM. For utilization at a pH of approximately 7, a concentration of 100 mM is often advantageous. In certain embodiments, the upper limit on amine or amine moiety concentration is determined by the maximum solubility of the amine or

moiety in the environment in which it is being used, for example in water. In general, the amount of amine or amine moiety employed is that which is sufficient to effect the transformation of the oxidized metal-containing ECL moiety into its excited state so that luminescence occurs. Those of ordinary skill in the art, equipped with the teachings herein, can determine empirically the amount of amine or amine moiety advantageously used for the particular system being analyzed, without undue experimentation.

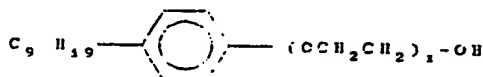
As described in PCT published application US 89/04915, entitled Enhanced Electrochemiluminescent Reaction, the contents of which are incorporated by reference, the assays of the invention are desirably carried out in the presence of an enhancer, typically a compound of the formula



wherein R is hydrogen or  $\text{C}_n\text{H}_{n+1}$ , R' is  $\text{C}_n\text{H}_{2n}$ , x is 0 to 70, and n is from 1 to 20. Preferably, n can be from 1 to 4. Specific examples are a substance available in commerce under the name Triton X-100, of the formula



wherein x is 9-10, and a substance available in commerce under the name Triton N-401 (NPE-40), of the formula



## 28.1

wherein x is 40. The enhancer is generally utilized in an amount sufficient so that in its presence the desired increase in emission of electromagnetic radiation occurs. Typically, the amount is .01% to 5.0%, more specifically 0.1% to 1.0%, v/v.

The ECL moiety used in accordance with the present invention is induced to emit electromagnetic

radiation by stimulating it into an excited state. This is accomplished by exposing the system in which the ECL moiety is incorporated to electrochemical energy. The potential at which oxidation of the ECL moiety and the species forming a strong reductant occurs depends upon the exact chemical structures thereof, as well as factors such as the pH of the system and the nature of the electrode used to introduce electrochemical energy. It is well known to those of ordinary skill in the art how to determine the optimal potential and emission wavelength of an electrochemiluminescent system. Certain preferred methods of stimulating the ECL system are disclosed in PCT published application US 89/01814, the contents of which are incorporated herein by reference.

#### Apparatus for Measuring Electrochemiluminescence

An apparatus for carrying out the assays of the invention is described in Figs. 1 and 2. Fig. 1 discloses an advantageous ECL apparatus, but the methods of the present invention are not limited to application in apparatus 10, but rather may be employed in other types of ECL apparatus which include a working electrode or other triggering surface to provide electrochemical energy to trigger the ECL moiety into electrochemiluminescence. While the methods of the invention can be carried out in a static or flow-through mode, apparatus 10 includes a flow-through cell, which provides distinct advantages for many types of samples including binding assay samples. Further details of apparatus for carrying out the ECL assays of the invention are disclosed in published PCT applications US 89/04854 and U.S. 90/01370.

Apparatus 10 includes an electrochemical cell 12, a light detection/measurement device 14, which

may advantageously be a photomultiplier tube (PMT), photodiode, charge coupled device, photographic film or emulsion or the like, and a pump 16, which is advantageously a peristaltic pump, to provide for fluid transport to, through and from cell 12. Alternatively, a positive displacement pump may be used. A shutter mechanism 18 is provided between cell 12 and PMT 14 and is controllably operated to open only so far as to expose PMT 14 to cell 12 during ECL measurement periods. The shutter mechanism may be closed, for example, during maintenance. Also included in apparatus 10 but not illustrated in Fig. 1 is a lightproof housing intended to mount the various components therein and to shield PMT 14 from any external light during the ECL measurements.

Cell 12 itself includes a first mounting block 20 through which passes an inlet tube 22 and an outlet tube 24, which may be advantageously constructed of stainless steel. Mounting block 20 has a first, outer surface 26 and a second, inner surface 28 defining one side of a sample-holding volume 30 of cell 12 in which cell 12 holds the cleaning and/or conditioning and/or measurement solutions during corresponding operations of apparatus 10. Inlet and outlet tubes 22, 24 pass through mounting block 20 from outer surface 26 to inner surface 28 and open into sample-holding volume 30. A second mounting block 32, advantageously constructed of stainless steel also has a first, outer surface 34 and a second, inner surface 36. Second mounting block 32 is separated from first mounting block 20 by an annular spacer 38, advantageously constructed of Teflon or other non-contaminable material. Thus, outer surface 34 of mounting block 32 defines part of the second side of the sample-holding volume 30. Spacer 38 has an outer portion 40 and a central aperture 42 whose inner edge

44 defines the side wall of sample-holding volume 30. Outer portion 40 seals the inner surface 28 of first mounting block 20 to outer surface 34 of second mounting block 32 to prevent any solution from passing out from sample-holding volume 30 between the two surfaces 28, 34. Mounting block 32 further has a central aperture 46 in which a window 48 is seal-fitted to define the rest of the second side of sample-holding volume 30 as a continuation of outer surface 34.

Window 48 is formed of a material which is substantially transparent at the wavelength of electrochemiluminescent light emitted by the ECL moiety. Window 48 is therefore advantageously formed of glass, plastic, quartz or the like.

Inlet tube 22 intersects sample-holding volume 30 at a first end 50 thereof adjacent to spacer 38 and outlet tube 24 intersects sample-holding volume 30 at a second end 52 thereof, adjacent spacer 38. The combination of inlet tube 22, sample-holding volume 30 and outlet tube 24 thereby provides a continuous flow path for the narrow, substantially laminar flow of a solution to, through and from cell 12. Arrows A and B represent the flow into and out of inlet tube 22 and outlet tube 24, respectively.

Mounted on inner surface 28 of first mounting block 20 is a working electrode system 54 which, in the illustrated embodiment, includes first and second working electrodes 56 and 58. In other embodiments, a single working electrode may advantageously be provided, or only electrode 56 may be a working electrode. Working electrodes 56, 58 are where the electrochemical and ECL reactions of interest can take place. Working electrodes 56, 58 are solid voltammetric electrodes and may therefore be advantageously constructed of platinum, gold, carbons or other materials which are effective for this

purpose. Wire connectors 60, 62 connected to working electrodes 56, 58, respectively, pass out through first mounting block 20.

5           Connectors 60, 62 are both connected to a first, "working electrode" terminal 64 of a voltage control 66, illustrated in Fig. 2. Voltage control 66 advantageously operates in the manner of a potentiostat to supply voltage signals to working electrodes 56, 58 and optionally to measure current flowing therefrom  
10           during an ECL measurement. Alternatively, connectors 60, 62 may be connected to separate terminals of voltage control 66 for individual operation.

          The potentiostat operation of voltage control 66 is further effected through a counter electrode 68  
15           and, optionally but advantageously, a reference electrode 70. In the illustrated embodiment, mounting block 32 is made of stainless steel and counter electrode 68 consists in exposed surfaces 72, 74 of mounting block 32. Counter electrode 72, 74 and  
20           working electrodes 56, 58 provide the interface to impress the potential on the solution within sample-holding volume 30 which energizes the chemical reactions and triggers electrochemiluminescence in the sample and/or provides energy for cleaning and  
25           conditioning the surfaces of cell 12. Counter electrode 72, 74 is connected by a wire connector 76 to a second, "counter electrode" terminal 78 of voltage control 66.

          Reference electrode 70 provides a reference  
30           voltage to which the voltage applied by the working electrodes 56, 58 is referred, for example, +1.2 volts versus the reference. Reference electrode 70 is advantageously located in outlet tube 24 at a position 80 spaced from cell 12 and is connected through a wire  
35           connector 82 to a third "reference electrode" terminal 84 of voltage control 66. In the three electrode mode,



current may not flow through reference electrode 70. Reference electrode 70 may be used in a three electrode mode of operation to provide a poised, known and stable voltage and is therefore advantageously constructed of silver/silver chloride (Ag/AgCl) or is a saturated calomel electrode (SCE). Voltage control 66 may be operable in a two electrode mode of operation using only working electrode 56 and electrode 58 as a counter/reference electrode. In this two electrode mode of operation, counter/reference electrode 58 is electrically connected to voltage control terminals 78 and 84 on voltage control 66. In this case, voltage control 66 operates essentially as a battery. Voltage control 66 supplies voltage signals to working and counter electrodes 56 and 58 and optionally measures the current flowing through the respective electrodes. Reference electrode 70 may alternatively be a so-called "quasi-reference" electrode constructed of platinum, gold, stainless steel or other material, which provides a less stable voltage, yet one that is measurable with respect to the solution in contact. In both the two and three electrode mode, the reference electrode 70 or 58 serves the purpose of providing a reference against which the voltage applied to working electrodes 56 is measured. The poised voltage reference is currently considered to be more advantageous. Voltage control 66 in its potentiostat operation controls the various electrodes by providing a known voltage at working electrodes 56, 58 with respect to reference electrode 70 while measuring the current flow between working electrodes 56, 58 and counter electrode 72, 74. Potentiostats for this purpose are well known, and the internal structure of voltage control 66 may therefore correspond to any of the conventional, commercially available potentiostats which produce the above-recited functions and so do not form a part of the present

invention per se. Indeed, apparatus 10 may alternatively be constructed without an internal voltage control 66, and may be adapted to be connected to an external potentiostat which is separately  
5 controlled for providing the required voltage signals to electrodes 56, 58, 72, 74 and 70. These voltage signals, applied in a specific manner as described below, provide repeatable initial conditions for the surfaces of working electrodes 56, 58 and  
10 advantageously for the surfaces of cell 12 as a whole, a feature which contributes significantly to improved precision in ECL measurements.

Pump 16 is advantageously positioned at outlet tube 24 to "pull" solution from a sample volume  
15 in the direction of arrow A into inlet tube 22. The solution will flow through inlet tube 22, sample-holding volume 30 and outlet tube 24 past reference electrode 70 and out in the direction of arrow B. Alternatively, pump 16 may be positioned at inlet tube  
20 22 to "push" the solution through apparatus 10. Advantageously, this same flow path through inlet tube 22, sample-holding volume 30 and outlet tube 24 is used for all solutions and fluids which pass through cell 12, whereby each fluid performs a hydrodynamic cleaning  
25 action in forcing the previous fluid out of cell 12. Pump 16 may be controlled to suspend its operation to hold a particular solution in cell 12 for any period of time.

The flow-through construction of apparatus 10  
30 permits working electrodes to be impressed with a variable voltage or to be continuously held at a preoperative potential while being continuously exposed to one or more solutions without exposing working electrodes 56, 58 (or counter and reference electrodes  
35 72, 74, 70) to air. Exposure to air, which opens the circuit to the reference electrode 70, permits unknown,

random voltage fluctuations which destroy the reproducibility of surface conditions on working electrodes 56, 58. The flow-through construction permits the rapid alternation between initializing steps, in which electrode system 54 is cleaned and conditioned, and measurement steps, in which one or more measurement waveforms or sweeps trigger ECL.

Figs. 23 and 24 schematically show a cell and magnets 27/37 which is equipped with a magnet system which advantageously imposes field lines which are largely parallel to the plane of the electrode surface 56, 58. The magnet system consists of a plurality of individual permanent or electromagnets which are stacked and oriented such that the north and south poles of the magnets 27/37 alternate in the stack. The individual magnets of magnets 27/37 are separated by air or any non-magnetically responsive material. The arrangement as shown in Figs. 23 and 24 advantageously applies magnetic lines of force to the region above the working electrode which are nearly horizontal to the plane of the electrode. This induces an orientation of the magnetically responsive particles in which the particles lie upon the surface of the electrode and are readily accessible to the electrochemical energy supplied by the electrode; see Fig. 20.

The magnet system 27/37 shown in Figs. 23 and 24 also is advantageous in that the magnetic field lines do not extend a long distance from the magnet structure; see Fig. 20. The magnetic field from such a magnet system is not likely, therefore, to induce permanent magnetic behavior or ferromagnetic materials near the electrode apparatus and will not severely affect the operation of a photomultiplier tube near the flow cell apparatus. The apparatus depicted in Fig. 25 is as shown in Figs. 1 and 2, and is as described above with respect to Figs. 1 and 2, except that in Fig. 25,

## 35.1

positioned vertically below the horizontally oriented electrode 56 or electrodes 56, 58 are a plurality of magnets 27/37 in north-south orientation as shown in Figs. 23 and 24.

- 5                   The invention is also directed to reagent compositions. Broadly, the reagent compositions may be any one of the components of the assay systems of the invention, i.e., (a) electrolyte, (b) label compound containing an ECL moiety, (c) particles, including
- 10                   magnetically responsive particles, (d) analyte of interest or an analog of the analyte of interest, (e) a binding partner of the analyte of interest or of its analog, (f) a reactive component capable of reacting with (d) or (e), (g) a reductant, or (h) an
- 15                   electrochemiluminescence-reaction enhancer. The reagents may be combined with one another for convenience of use, i.e., two component, three component, and higher multiple component mixtures may be prepared, provided that the components are not reactive
- 20                   with one another during storage so as to impair their function in the intended assay. Desirably, the reagents are two-component or multicomponent mixtures which contain particles as well as one or more other components.
- 25                   The invention is also directed to kits. The kits may include vessels containing one or more of the components (a) to (h) recited above or the kits may contain vessels containing one or more reagent compositions as described above comprising mixtures of
- 30                   those components, all for use in the assay methods and systems of the invention.

Description of Preferred Embodiments of the Invention

While a wide range of particles can be employed in the particle-based assays of the invention, generally the particles have a density of from 1.0 to 5.0 g/mL and preferably have a density of from 1.1 to 2 g/mL. Choice of the optimum density is within the skill of the art, the rate of settling in gravity-driven assays being a trade-off between the speed of the assay and the desire to create a uniform layer of complex on the electrode surface.

Particles having a wide range of mean diameters can also be employed. Particles having a mean diameter of from 0.001 to 200  $\mu\text{m}$  such as 0.05 to 200  $\mu\text{m}$  can be used; and preferably the particles have a mean diameter of from 0.01 to 10  $\mu\text{m}$ .

Wide ranges of concentration of particles in the assay composition can also be employed. For example, the concentration can range from 1 to 10,000  $\mu\text{g/mL}$  to preferably from 5 to 1000  $\mu\text{g/mL}$ . Desirably, the density of the particles, their size and their concentration is selected such that the particles settle at a rate of at least 0.5 mm/min and preferably at a faster rate.

In the filtration mode of performing the invention, the filtration means desirably has a pore size, measured as mean diameter, from broadly 0.01 to 90% of the mean diameter of the particles and preferably from 10% to 90% of that diameter.

The art has described a number of magnetic particles which can be used in the assays of the invention. For example, U.S. Patent No. 4,628,037, 4,695,392, 4,695,393, 4,698,302, 4,554,088, U.K. Patent Application GB 2,005,019A and EP 0,180,384, all of which are incorporated herein by reference, describe a variety of magnetic particles which can be used with

success. The particles may be paramagnetic or ferromagnetic and may be coated with various materials to which binding compounds are coupled so that the magnetic particle can be used in immunoassays.

5 Desirably the magnetic particles used in the invention have a susceptibility of at least 0.001 cgs units and desirably the susceptibility is at least 0.01 cgs units. The magnetic particles may have a broad range of densities, i.e. from substantially less than that of  
10 water, 0.01, to 5 g/mL and preferably from 0.5 to 2 g/mL. The particle sizes can range from 0.001 to 200, such as 0.001 to 200 or 0.05 to 200  $\mu\text{m}$ ; and preferably from 0.01 to 10  $\mu\text{m}$ . The concentration of the particles may range broadly from 1 to 10,000  $\mu\text{g}$  per mL and  
15 preferably is from 5 to 1000  $\mu\text{g}$  per mL.

Desirably the magnetic particles which are used have a low magnetic resonance, as described for example EP 0,180,384, so that after the magnetic field is removed from the electrode surface, the particles  
20 demagnetize and can be swept out of the assay cell. Desirably the density, concentration and particle size of the magnetic particles is chosen such that the settling time is at least 0.5 mm/min and desirably it is above that rate. In operation of the magnetic cell  
25 it is often desirable to remove the magnet means from the electrode surface prior to inducing electrochemiluminescence in order not to interfere with the operation of the photomultiplier tube.

### 30 Assays

A variety of assays can be performed using the methods of the invention.

An assay was performed as shown in Fig. 3.  
35 The PCR products resulting from the reaction were labeled with biotin and an ECL label (tris (2,2'

bipyridine) Ru II,  $\text{Ru}(\text{bpy})_3^{2+}$ ). Streptavidin beads captured the bifunctionalized DNA via biotin streptavidin binding and this was followed by washing. The bead bound product was then subjected to analysis detecting the ECL label.

An assay was performed as shown in Fig. 4. The biotinylated PCR product was captured on streptavidin beads and the non-biotinylated strand removed. The bead bound PCR product was then hybridized with an ECL labeled  $(\text{Ru}(\text{bpy})_3^{2+})$ -oligonucleotide. This was followed by ECL analysis to detect the label.

An assay was conducted as shown in Fig. 5. The hybrids were captured on streptavidin beads. This was followed by ECL analysis without washing.

An assay was conducted and the results are shown in Fig. 6. The assay was for the presence of HPV 16 and 18 using DNA samples isolated from the cell lines SiHa and HeLa positive for both virus types and oligonucleotides specific for each virus type. The primers 2PV16, 2PV18 were biotinylated and 3PV16, 3PV18 were ECL-labeled-oligonucleotides. The 2/3PV16 and 2/3PV18 oligonucleotides were specific for HPV 16 and 18 respectively. The resultant bead captured ECL label was analyzed for ECL using an analyzer as described in Fig. 1. The results were plotted as ECL counts for each sample primer combination.

An assay was conducted and the results are shown in Fig. 7. The resultant bead bound ECL label was analyzed for ECL using an analyzer as described in Fig. 1. The ECL peak photon counts were plotted against increasing concentrations HPV 16 DNA, expressed as a ratio of viral copies to total cellular DNA copies. The primers used in this analysis for HPV16 were 1PV16 (biotin label) and 2PV16 (ECL label). DNA

used for each PCR was maintained at a constant 1 $\mu$ g using calf thymus DNA.

An assay was conducted and the results are shown in Fig. 8. The PCR was performed using  
5 biotinylated HRP2 with unlabeled HRP1 (for probes 1T24 and 1CHR) and biotinylated HRP1 with unlabeled HRP2 (for probes 2T24 and 2CHR), generating bead bound single stranded targets for hybridization. The DNA  
10 samples were the normal (Placenta) Ha-ras Gene and the mutant (NIH3T3-T24) Ha-ras Gene. The hybridization of the bead bound DNA with ECL label-1T24 (1T24), ECL label-2T24 (2T24), ECL label-1CHR (1CHR) and ECL label-2CHR (2CHR) was followed by TEMAC washes. Resultant  
15 bead bound ECL label was analyzed for ECL using an analyzer as described in Fig. 1. Results were plotted as ECL counts for each sample probe combination.

An assay was conducted and the results are shown in Fig. 9. The PCR was performed as described in Fig. 8 using only biotinylated HRP2 with unlabeled HRP1  
20 (for probes 1T24 and 1CHR). The probes used were: 1T24 and 1CHR containing P<sup>32</sup> (1T24-P, 1CHR-P) as controls. With the 1T24 and 1CHR containing both P<sup>32</sup> and ECL label to determine the effects of the ECL label. The samples were washed as earlier with TEMAC. The resultant bead  
25 bound P<sup>32</sup> was analyzed on addition of scintillation cocktail in a scintillation counter. The results were plotted as P<sup>32</sup> counts per second for each sample probe combination.

An assay was conducted and the results are shown in Fig. 10. The assay was performed as described  
30 in Fig. 4. The sample was placental DNA and amplification was performed using biotinylated HRP2 with unlabeled HRP1 (for probes 1T24 and 1CHR). The resultant PCR product was then sampled to give a set of  
35 samples containing differing amounts of product. These sets of samples were then hybridized with either probes



labeled with  $P^{32}$  (1T24-P32 and 1CHR-P32) or ECL label (1T24-ECL and 1CHR-ECL). The results from each studies were then normalized using the average peak value from each label for the 90 $\mu$ l sample. These normalized figures allow a more effective comparison of signal to background and the comparative response of the two methods. The inset figure illustrates the response at the lower level of the dilution curve. The samples were handled as described earlier. (Fig. 6 and Fig. 8.)

An assay was conducted and the results are shown in Fig. 11. The PCR was performed using biotinylated 2PV18 and unlabeled 1PV18 using HeLa DNA (400 copies per cell) using the PCR format illustrated in Fig. 3. The resultant PCR reaction was then hybridized with the specific probe ECL label-3PV18. The hybridization mixture was then added to streptavidin coated beads and the resultant bead bound ECL label was directly analyzed for ECL using an ECL analyzer as described in Fig. 1. The results were plotted as ECL counts verses HPV18 copies added to the PCR.

The following non-limiting Examples are given by way of illustration and are not to be considered a limitation of this invention, many apparent variations of which are possible without departing from the spirit or scope thereof.

#### EXAMPLES

##### Instrumentation, Materials, and Methods

###### (1) Instrumentation

A flow-through apparatus, employing three electrodes, as described in Figs. 1 and 2, was used.

Working Electrode -- Au disk, 3 mm diameter  
Counter Electrode -- Au disk, 3 mm diameter  
Reference Electrode -- Ag/AgCl

Teflon Gasket (0.15"thick)

Plexiglas Faceplate

Inlet Tubing = .042" id polypropylene

Aspiration Rates:variable from 0.01 to 5 mL/min

5 Potentiostat: microprocessor controlled

Luminometer using Hamamatsu R374 PMT (low gain red sensitive tube); PMT Voltage variable 0-1400 V

(2) Materials

- 10 (a) ECL Label:  $\text{Ru}(\text{bpy})_3^{2+}$
- (b) ECL Buffer: 112 mM  $\text{KH}_2\text{PO}_4$ , 88 mM  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , 50  $\mu\text{M}$  NaCl, 6.5 mM  $\text{NaN}_3$ , 0.8  $\mu\text{M}$  Triton X-100, 0.4 mM Tween 20, 100 mM tripropylamine in  $\text{H}_2\text{O}$
- 15 (c) ECL Diluent: 37.5 mM  $\text{KH}_2\text{PO}_4$ , 109.2 mM  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , 151.7 mM NaCl, 0.65 mM  $\text{NaN}_3$ , 0.43 mM bovine serum albumin in  $\text{H}_2\text{O}$
- 20 (d)  $\text{Ru}(\text{bpy})_3^{2+}$ -NHS:  $\text{Ru}(2,2'\text{-bipyridyl})_2(4\text{-}[3\text{-(1,3-dioxolan-2-yl)propyl}]\text{-4'-methyl-2,2'-bipyridine})^{2+}$
- (e) Dynal Particles:
- 25 (i) Dynal M-450 Dynabeads, 4.5  $\mu\text{m}$  diameter superparamagnetic particles, 30 mg/mL, obtained from Dynal, 45 North Station Plaza, Great Neck, NY 11021
- 30 (ii) Dynal M-280 Dynabeads, 2.8  $\mu\text{m}$  diameter superparamagnetic particles, 10 mg/mL, obtained for Dynal, 45 North Station Plaza, Great Neck, NY 11021

(3) ECL Measurement Cycle (three electrode cell operation)

The ECL measurement cycle consists of three steps: (1) preconditioning, (2) measuring, and (3) cleaning. The preconditioning step involves the application of a voltage triangle wave of 0.0 V to +2.2 V to -1.0 V to +0.6 V at 2.0 V/sec. The measurement step involves the application of a triangle waveform from +0.6 V to +2.8 V to +2.0 V at 1.0 V/s. The cleaning step involves the application of a voltage square wave from +0.0 V to +3.0 V to -0.5 V to 0.0 V. All voltages are relative to the Ag/AgCl reference electrode.

15

EXAMPLE 1

Apparatus and Method for Collection of Microparticles by Gravity

The measurement is conducted in a cell as shown in Fig. 12. References made to Fig. 12 which depict an apparatus for conducting an assay using the force of gravity. The components of the apparatus include a transparent window identified by reference numeral 48, a gasket identified by reference numeral 222, a block 20 which includes an inlet 22, a working electrode 56/58, a counterelectrode 72/74 and an outlet port 24. The plane of the cell block is horizontal, i.e. perpendicular to the direction of the earth's gravitational field. Labeled microparticles (Dynal) in an ECL buffer are drawn to the cell by means of a peristaltic pump. The pump is turned off after the particles reach the cell. The microparticles in the cell chamber fall onto the working electrode surface. The rate of fall of microparticles is determined to be approximately constant at 0.5 mm/min over a distance of 10 mm, as shown in Fig. 13. The number of particles to settle is a function of time and rate of fall. The

ECL intensity is proportional to the number of particles that settle on the working electrode. The number of particles that reach the surface, and therefore the ECL intensity is limited by the height of fluid sample over the working electrode. Fig. 14 shows the ECL intensity as a function of deposition time for two cells of different gasket thicknesses, 0.015 and 0.075 inches, respectively. Both cells have similar rates of deposition of microparticles but the cell with a thicker gasket gives an maximum reading which is five times larger. The results of an AFP (alpha fetal protein) assay is shown in Fig. 15, comparing the two cells. Again, the cell with the thicker gasket produces five times the ECL signal intensity.

#### EXAMPLE 2

##### ECL Apparatus and Method for Deposition of Microparticles.

Magnetic Collection using a Sedimentation Cell.

A cell for conduct of an assay using magnetic force to cause the microparticulate to settle is shown in Fig. 16. Reference numeral 48 refers to a transparent window, reference numeral 122 to a gasket, reference numeral 22 to the inlet in the cell block, reference numeral 56/58 to the working electrode, reference numeral 24 to the sample outlet, reference numeral 20 to the cell block itself and reference 27 to an electromagnet.

The plane of the cell block is oriented horizontally. Labeled microparticles (Dynal) in ECL buffer are drawn to the cell by means of a peristaltic pump. The pump is turned off after the microparticles reach the cell. The microparticles in the cell chamber are drawn to the working electrode by means of a magnetic field generated using electromagnet 27

operating at 12 volts and 1.5 amps. By application of the electromagnet, the rate of deposition of microparticles is greatly increased over that observed when the microparticles settle solely due to the force of gravity. This is shown in Fig. 17.

### EXAMPLE 3

#### ECL Apparatus and Method for Deposition of Microparticles.

##### 10      Magnetic Collection using a Collection Cell.

An assay is carried out in a cell as described in Fig. 18. With reference to Fig. 18, reference numeral 48 refers to transparent window, 15 reference numeral 132 to a gasket, reference numeral 22 to an inlet in the cell block, reference numeral 56/58 to a working electrode, reference numeral 20 to the cell block itself, reference numeral 24 to the sample outlet and reference numeral 37 to a permanent magnet.

20      The plane of the cell block is oriented horizontally. Labeled microparticles (Dyna) in ECL buffer are drawn to the electrochemical cell by means of a peristaltic pump. Prior to the sample introduction, permanent magnet 37 is positioned 25 immediately below the working electrode/solution interface at a distance of 0.035 inches. As the sample is being drawn to the cell, the microparticles deposit onto an area over the working electrode, as defined by the area of the magnet. The pump is turned off and the 30 magnetic withdrawn after the entire sample is deposited. The longer the collection time, the more particles are deposited. Increasing the concentration of particles on the working electrode results in an increased ECL intensity as shown in Fig. 19.

35

EXAMPLE 4Use of Magnet for Deposition of Microparticles.  
Magnetic Field Orientation.

5                    Microparticles 96, 96' which are attracted to  
a magnet 27/37, whether it be a permanent magnet or  
electromagnet, align with the orientation of the  
magnetic field 98, 98', such as in Fig. 20 which  
10                   depicts magnetic fields 98 and 98', and the resultant  
particle arrangements 96 and 96' which are parallel (A)  
and perpendicular (B) to the surface of the working  
electrode 56/58, in the vicinity of that surface.

EXAMPLE 515                   Particle Collection and Concentration by Filtration

                    Microparticles which are magnetically  
responsive, non-magnetically responsive, and of a wide  
range of densities can advantageously be collected by  
20                   filtration upon the surface of a membrane filter. In  
one embodiment of the invention, the particles are  
pumped through a portion of a filter membrane which has  
pore sizes which are smaller than the diameter of the  
particles but preferably are substantially smaller than  
25                   the particle diameter and at a sufficiently high  
surface density such that the collection of particles  
will not cause blockage of the pores. The filter is  
advantageously largely transparent such that the  
filter, after collection of the particles, can be  
30                   placed upon the surface of a working electrode for the  
purpose of inducing ECL from the particles and  
measuring the luminescence to measure the quantity of  
ECL label on the particles.

                    In another embodiment, the membrane filter  
35                   having pore sizes as described above is attached or  
placed upon the surface of an absorbent material such

that capillarity or "wicking" will spontaneously draw fluids containing microparticles through the membrane filter without requiring any apparatus to induce the flow of fluid through the filter.

5 In the preferred embodiment, the membrane filter, having pore sizes as described above, is coated with a thin film of metal or other electronically conductive material such that the surface of the membrane can serve as a working electrode in an ECL  
10 apparatus. The conductive films are readily applied to the surface of a membrane by methods commonly used in the fabrication of microelectronic devices, e.g., thermal evaporation or sputtering. Such a filter-electrode is readily mounted in a flow cell such that  
15 the flow-path for the fluid is through the filter-electrode. Particles in the stream are trapped by the filter-electrode and are easily washed *in-situ* providing for a rapid and simple means for performing heterogeneous assays without any external washing  
20 apparatus.

#### EXAMPLE 6

##### Particle Collection and Concentration by Centrifugal Method

25 The rotary flow cell shown in Fig. 21 provides another means to capture the complex on the surface of the working electrode in order to measure luminescence. The assay solution enters the apparatus  
30 via inlet 261 and is pumped into cell 262 through rotary seal 263 while a rotational motion is imparted to the cell, as indicated by arrow R. The denser particles of the complex are concentrated on the surface of working electrode 264. While the cell is  
35 still rotating the solution passes out of the cell via outlet 268. The light output passing through cell

window 267 is measured by photomultiplier tube 265. The light output is directed from the vertical working electrode surface(s) 264 reflecting off curved mirror surface(s) 266 located at the center of the cell; counter electrode(s) 269 is also shown. The cell is then flushed and cleaned for the next cycle. This may be accomplished with the cell stopped or rotating. Thus, Fig. 21 shows a centrifugal method and apparatus of the invention for capturing particles, as well as a centrifugal flow cell of the invention.

#### EXAMPLE 7

##### Coating of Particles With Labeled Non-specific Protein at Moderate Surface Concentration

30 mg (1 ml) of 4.5  $\mu$ m uncoated magnetically responsive, polystyrene M-450 DYNABEADS (DYNAL, Oslo, Norway) were washed by magnetic separation with a 150 mM phosphate buffer pH 7.5 solution using 2 ml/wash. 150  $\mu$ g of Ru(bpy)<sub>3</sub><sup>2+</sup>-labeled mouse IgG (Jackson Immunochemicals) in 1 ml of phosphate buffer saline (PBS) with 0.05% thimerasol were added to the particles. This mixture was allowed to incubate overnight at room temperature with rotation. The solution was then magnetically separated from the particles and removed. To block unreacted sites, 1 ml of 3% BSA/PBS with 0.05% sodium azide was added to the particles, and the resultant solution was allowed to incubate 2 hours at room temperature. The particles were washed 5 times (2 ml/wash), and then finally resuspended in 6 ml of the same buffer for storage.



EXAMPLE 8Electrochemiluminescent (ECL) Measurement Using  
Magnetically Responsive Particles

5                   Uniform and nonuniform, polymeric and non-  
polymeric, magnetically responsive particles (Dynal,  
Oslo, Norway; Polysciences, Warrington, PA 18976;  
Cortex Biochem, San Leandro, CA 94577; Aldrich,  
Milwaukee, WI 53201) were coated with labeled proteins  
10 as described in Example 7. The coated particles were  
washed with ECL buffer three times before making 2 mL  
of a 300 µg/mL suspension. Using a peristaltic pump,  
500 µl of the particle suspension was drawn into the  
flow cell (Example 3). As the particles flowed to the  
15 working electrode, they were attracted and concentrated  
onto the working electrode surface by a magnet.  
Electrochemiluminescence using the magnetic particles  
was measured using a Hamamatsu R374 photomultiplier  
tube centered above the flow cell where particles had  
20 concentrated on the working electrode surface. Table I  
shows ECL photoemission levels obtained from the  
labeled-protein coated magnetically responsive  
particles.

25

Table I.

5 ECL Measurements from Different Magnetically Responsive  
Particles

10	Particle Type	Diameter ( $\mu\text{m}$ )	Density (g/mL)	Material	ECL Counts
15	Glass	8.0	2.4	soda lime glass	2200
		2.0	2.4	soda lime glass	8500
20	Quartz	0.3 - 3.5	2.5	SiO <sub>2</sub>	1150
	Gold	1.0 - 2.0	19.3	Au	1100

25

EXAMPLE 9

Electrochemiluminescent (ECL) Measurement Using  
Nonmagnetic Particles

30 Uniform and nonuniform, polymeric and nonpolymeric, non-magnetical responsive particles (Aldrich, Milwaukee, WI 53201; Duke Scientific, Palo Alto, CA 94303) were coated with labeled proteins as described in Example 7. The coated particles were washed

35 with ECL buffer three times before making 2 mL of a 300  $\mu\text{g/mL}$  suspension. Using a peristaltic pump, 500  $\mu\text{L}$  of the particle suspension was drawn into the flow cell. The coated particles were then concentrated onto the working electrode by gravitational means as described in

40 Example 1. Electrochemiluminescence using the nonmagnetic particles was measured with a Hamamatsu R374 photomultiplier tube centered above the flow cell where particles had concentrated on the working electrode surface. Table II shows ECL photoemission levels

45 obtained from the coated nonmagnetic particles.

Table II.

ECL Measurement from non-magnetically responsive  
Particles by Gravity Collection

Particle Type	Diameter ( $\mu\text{m}$ )	Particle Density (g/mL)	Material	ECL Counts
Rhône-Poulenc	4.0	1.5	Polystyrene Divinyl Benzene/ $\text{Fe}_3\text{O}_4$	1680
	1.5-2.1	1.4	Polystyrene/ $\text{Fe}_3\text{O}_4$	462
Poly-sciences	1.5-2.1	2.1	Polystyrene/ $\text{FeO}_2$	504
Dynal	4.5	1.5	Polystyrene/ $\text{Fe}_2\text{O}_3$	4200
Cortex	1.0-10	1.3	Cellulose/ $\text{Fe}_3\text{O}_4$	125
	1.0-10	1.8	Polyacrolein/ $\text{Fe}_3\text{O}_4$	125
	1.0-25	1.2	Polyacrylamide/ $\text{Fe}_3\text{O}_4$ w/ charcoal	125
Nickel	3.0	8.9	Ni	125

EXAMPLE 10

Preparation of Physically Adsorbed Sheep Anti-Thyroid  
Stimulating Hormone (TSH) Coated Dynal Particles

5

(REAGENT I)

10      1mL of 4.5 $\mu\text{m}$  uncoated magnetic, polystyrene particles with -OH residues on their surface (DYNAL, DYNABEADS M-450, DYNAL A.S. Oslo, Norway) was washed by magnetic separation with a 150mM sodium carbonate / bicarbonate pH 9.6 solution using 2 mL/wash. 0.5mg of affinity purified Sheep anti-TSH, HCG scrubbed antibody

(CIBA) in 1 mL of the carbo/bicarbo solution was added to the particles. This mixture was incubated overnight at room temperature with mixing. The solution was then magnetically separated from the particles and removed.

5 1mL of 3% BSA/PBS w/ 0.05% sodium azide was added and incubated 2 hours at room temperature with agitation to block unreacted sites. The particles were washed 5 times (2mL/wash) then finally resuspended in 1mL of the same buffer for storage. The final concentration of

10 Bead Reagent I was 3% by weight.

#### EXAMPLE 11

#### Preparation of Ouabain-BSA Conjugate

#### (REAGENT II)

15

#### ACTIVATION OF OUABAIN:

60.4mg of ouabain octahydrate (Aldrich Cat# 14,193-3) in 6mL of deionized (di) H<sub>2</sub>O (wrapped in foil) was mixed with 87mg of sodium metaperiodate

20 (Mallinckrodt Cat# 1139) and the mixture was incubated at room temperature for 2 hours, rotating. The reaction was terminated by passing the reaction mixture through Dowex 1 X 8 - 50 ion exchange resin (Aldrich Cat# 21,740-9) with diH<sub>2</sub>O. 200μL 1M sodium phosphate

25 pH 7.2 was added to adjust the pH of the solution to 7.0.

#### CONJUGATION OF ACTIVATED OUABAIN TO BSA:

50mg of activated ouabain(4.6mL) was then added dropwise to 108mg bovine serum albumin BSA, Miles

30 Fraction V) in 5mL 0.15M PBS pH 7.8. This is a 40:1 (OUABIN:BSA) ratio. The reaction was incubated at room temperature for 2 hours, mixing, followed by rapid addition of 30mg of sodium cyanoborohydride while

35 mixing. Free ouabain and excess sodium cyanoborohydride were removed by dialysis at 4°C in

0.15M PBS w/ 0.05% sodium azide pH 7.8. The Ouabain-BSA Conjugate Reagent II was stored at 4°C.

#### EXAMPLE 12

5     Preparation of Physically Adsorbed Ouabain-BSA Coated  
          Dynal Particles  
          (REAGENT III)

10         5mg of 4.5µm uncoated magnetic, polystyrene  
          particles with -OH residues on their surface (DYNAL,  
          DYNABEADS M-450, DYNAL A.S. Oslo, Norway) were washed  
          by magnetic separation with a 150mM sodium carbonate /  
          bicarbonate pH 9.6 solution using 10mL/wash. 3mg of  
15         Ouabain-BSA conjugate (Conjugate Reagent II) in 5 mL of  
          the carb/bicarb solution was added to the particles.  
          This mixture was incubated overnight at room  
          temperature while rotating. The solution was then  
          magnetically separated from the particles and removed.  
          5mL of 3% BSA/PBS w/ 0.05% sodium azide was added and  
20         incubated 2 hours at room temperature, rotating to  
          block unreacted sites. The particles were washed 5  
          times (10mL/wash) then finally resuspended in 1mL of  
          the same buffer for storage. The final concentration  
          of Bead Reagent III was 3% by weight.

25

#### EXAMPLE 13

Preparation of Ru(bpy)<sub>3</sub><sup>2+</sup>-Labeled Mouse Anti-Digoxin  
          (REAGENT IV)

30         1mg of mouse anti - Digoxin (Cambridge  
          Medical Technologies Cat# 200-014 Lot A3575) was  
          labeled with Ru(bpy)<sub>3</sub><sup>2+</sup>. The monoclonal antibody (MAb)  
          anti Digoxin antibody was buffer exchanged using  
          Centricon 30 microconcentrators (Amicon) into 0.15M  
35         potassium phosphate buffer, 0.15M NaCl pH 7.8, the  
          final volume being 0.5mL. Immediately prior to use,

0.5mg of  $\text{Ru}(\text{bpy})_3^{2+}$ -NHS was dissolved with 125 $\mu\text{L}$  of anhydrous dimethyl sulfoxide (Aldrich). To achieve a 25:1 molar ratio of  $\text{Ru}(\text{bpy})_3^{2+}$  to protein based on molecular weights of 1057 and 150,000 respectively, 0.18mg  $\text{Ru}(\text{bpy})_3^{2+}$ -NHS (45 $\mu\text{L}$ ) was added to the protein solution while shaking. The reaction tube was incubated in the dark at room temperature, 30 minutes, while shaking. The reaction was terminated by the addition of 25 $\mu\text{L}$  of 1M glycine and incubated for 10 minutes. The reaction mixture was purified by passage through a Sephadex G - 25 column (1 X 20cm in 0.15M potassium phosphate, 0.15M NaCl with 0.05% sodium azide pH 7.2). The  $\text{Ru}(\text{bpy})_3^{2+}$ -labeled mouse anti-digoxin fractions were collected and pooled. The labeled protein (Reagent IV) was determined to have 12 labels per protein molecule.

#### EXAMPLE 14

#### Preparation of $\text{Ru}(\text{bpy})_3^{2+}$ -Labeled Mouse Anti-Thyroid Stimulating Hormone (TSH) (REAGENT V)

0.5mg of mouse anti -TSH (CIBA) was labeled with  $\text{Ru}(\text{bpy})_3^{2+}$ . The MAb anti TSH antibody was buffer exchanged using Centricon 30 microconcentrators (Amicon) into 0.15M potassium phosphate buffer, 0.15M NaCl pH 7.8, the final volume being 0.35mL. Immediately prior to use, 0.5mg of  $\text{Ru}(\text{bpy})_3^{2+}$ -NHS was dissolved in 75 $\mu\text{L}$  of anhydrous dimethyl sulfoxide (Aldrich). To achieve a 50:1 molar ratio of  $\text{Ru}(\text{bpy})_3^{2+}$  label to protein based on molecular weights of 1057 and 150,000 respectively, 0.176mg  $\text{Ru}(\text{bpy})_3^{2+}$ -NHS (26.4 $\mu\text{L}$ ) was added to the protein solution while shaking. The reaction tube was incubated in the dark at room temperature, 30 minutes, while shaking. The reaction was terminated by the addition of 25 $\mu\text{L}$  of 1M glycine

and incubated for 10 minutes. The reaction mixture was purified by passage through a Sephadex G - 25 column (1 X 20cm in 0.15M potassium phosphate, 0.15M NaCl with 0.05% sodium azide pH 7.2). The Ru(bpy)<sub>3</sub><sup>2+</sup>-labeled mouse anti-TSH fractions were collected and pooled. The labeled protein (Reagent V) was determined to have 14 labels per protein.

#### EXAMPLE 15

##### One Step Separation Sandwich Assay for Thyroid Stimulating Hormone (TSH)

100μL serum calibrators (London Diagnostics TSH LumiTAG Kit), 25μL Ru(bpy)<sub>3</sub><sup>2+</sup>-labeled mouse anti-TSH (Reagent V) in ECL buffer and 25μL Sheep anti-TSH-DYNAL particles (Reagent I) in ECL buffer were combined and incubated in polypropylene tubes for 15 minutes, at room temperature, with mixing. The particles were then washed by magnetic separation and then resuspending the particles in 500μL of ECL buffer. This wash procedure was repeated two additional times. Finally, the particles were resuspended in 1mL of ECL buffer. The electrochemiluminescence (ECL) for each sample was read as described in Example 3. The ECL counts are directly proportional to the concentration of analyte present in the sample (increasing counts as the concentration of analyte increases). Table III demonstrates a representative assay curve.

**Table III.**

##### One-Step Separation Sandwich Assay; Detection of TSH

	TSH Concentration (μIU/mL)	ECL Counts (Duplicate Samples)	
	0.00	1918	1885
	0.05	2584	2530
	0.10	3365	3288
	0.50	8733	8652
	2.50	35688	35347
	10.0	125316	136994
	25.0	300248	288272
	50.0	549034	564948

EXAMPLE 16One Step Non Separation Sandwich Assay for Thyroid  
Stimulating Hormone (TSH)

5                   100 $\mu$ L serum calibrators (London Diagnostics  
TSH LumiTAG Kit), 25 $\mu$ L Ru(bpy)<sub>3</sub><sup>2+</sup>-labeled mouse anti-TSH  
(Reagent V) in ECL buffer and 25 $\mu$ L Sheep anti-TSH-DYNAL  
10 particles (Reagent I) in ECL buffer were combined and  
incubated in polypropylene tubes for 15 minutes, at  
room temperature, with mixing. Prior to reading  
results, 1mL of ECL buffer was added. The  
electrochemiluminescence (ECL) for each sample was read  
as described in Example 3. The ECL counts are directly  
proportional to the concentration of analyte present in  
15 the sample (increasing counts as the concentration of  
analyte increases). Table IV demonstrates a  
representative assay curve.

**Table IV.**  
One-Step Non-Separation Sandwich Assay:  
Detection of TSH

TSH Concentration ( $\mu$ IU/mL)	ECL Counts (Duplicate Samples)	
0.00	2610	2769
0.05	2870	2894
0.10	2970	2950
0.50	3473	3403
2.50	5588	5495
10.0	13051	13139
25.0	26468	27306
50.0	47104	48664



EXAMPLE 17Two Step Separation Competitive Assay for Digoxin

50 $\mu$ L serum calibrator (TDx Assay, Abbott Labs, Chicago, IL) and 25 $\mu$ L Ru(bpy)<sub>3</sub><sup>2+</sup>-labeled mouse anti-digoxin (Reagent IV) in ECL buffer, were combined and incubated 20 minutes at room temperature with mixing. 25 $\mu$ L Ouabain-BSA-DYNAL particles (Reagent III) in ECL buffer was added and incubated an additional 20 minutes, at room temperature, with mixing. The particles were then washed by magnetic separation and then resuspending the particles in 500 $\mu$ L of ECL buffer. This wash procedure was repeated two additional times. Finally, the particles were resuspended in 1mL of ECL buffer. The electrochemiluminescence (ECL) for each sample was read as described in Example 3. The ECL counts are inversely proportional to the concentration of analyte present in the sample (decreasing counts as the concentration of analyte increases). Table V demonstrates a representative assay curve.

**Table V.**  
Two-Step Separation Competitive Assay:  
Detection of Digoxin

Digoxin Concentration (ng/mL)	ECL Counts (Duplicate Samples)	
0.0	22031	21154
0.5	13367	13638
1.0	9506	9607
2.0	5244	5129
3.0	2959	2994
5.0	1581	1631

EXAMPLE 18Two Step Non Separation Competitive Assay for Digoxin

50 $\mu$ L serum calibrator (TDx Assay, Abbott  
5 Labs, Chicago, IL) and 25 $\mu$ L Ru(bpy)<sub>3</sub><sup>2+</sup>-labeled mouse  
anti-digoxin (Reagent IV) in ECL buffer, were combined  
and incubated 20 minutes at room temperature with  
mixing. 25 $\mu$ L Ouabain-BSA-DYNAL particles (Reagent III)  
10 in ECL buffer was added and incubated an additional 20  
minutes, at room temperature, with mixing. Prior to  
reading, the particles were resuspended in 1mL of ECL  
buffer. The electrochemiluminescence (ECL) for each  
sample was read as described in Example 3. The ECL  
15 counts are inversely proportional to the concentration  
of analyte present in the sample (decreasing counts as  
the concentration of analyte increases). Table VI  
demonstrates a representative assay curve.

Table VI.

Two-Step Non-Separation Competitive Assay:  
Detection of Digoxin

Digoxin Concentration (ng/mL)	ECL Counts (Duplicate Samples)	
0.0	42051	39643
0.5	28721	28074
1.0	22190	21364
2.0	14660	14542
3.0	11315	11893
5.0	9161	8945

EXAMPLE 19Two Step Non Separation Competitive Assay for Digoxin  
Using a Read Cycle With Additional Washing of Final  
Reaction Sample on the Electrode

5  
10  
15

50 $\mu$ L serum calibrator (TDx Assay, Abbott Labs, Chicago, IL) and 25 $\mu$ L Ru(bpy)<sub>3</sub><sup>2+</sup>-labeled mouse anti-digoxin (Reagent IV) in ECL buffer, were combined and incubated 20 minutes at room temperature with mixing. 25 $\mu$ L Ouabain-BSA-DYNAL particles (Reagent III) in ECL buffer was added and incubated an additional 20 minutes, at room temperature, with mixing. Prior to reading, the particles were resuspended in 1mL of ECL buffer. The electrochemiluminescence (ECL) for each sample was read as described in Example 3. The ECL counts are inversely proportional to the concentration of analyte present in the sample (decreasing counts as the concentration of analyte increases). Table VII demonstrates a representative assay curve.

**Table VII.**  
Two-Step Separation Competitive Assay:  
Detection of Digoxin

Digoxin Concentration (ng/mL)	ECL Counts (Duplicate Samples)	
0.0	42613	35309
0.5	24211	24168
1.0	17561	17206
2.0	10491	9909
3.0	6712	7145
5.0	4680	4603

EXAMPLE 20Oligonucleotide Synthesis

The oligonucleotides were made on an Applied Biosystems automated oligonucleotide synthesizer using the 8-cyanoethyl phosphoramidite (1). Oligonucleotide amino modifications to the 5' end occurred at the last coupling step, and at the 3' end by using a modified solid phase (controlled pore glass). Clontech (San Diego CA) supplied the amino modifiers. The resulting 5' modified oligonucleotides all contain a six carbon spacer arm to the amino group designated (C6, NH<sub>2</sub>). The 3' modified oligonucleotides all contain a three carbon spacer to the amino group. Oligonucleotides constructed, their modifications and utility are described below.

Oligonucleotides for the HPV study were directed to the E6 region as previously described (2).

The oligonucleotide sequences were as follows:

20 HPV 16; 1PV16 5' (C6, NH<sub>2</sub>) TTAGTGAGTATAGACATTATTGTTATAGTT;  
2PV16 5' (C6, NH<sub>2</sub>) CAGTTAATACACCTAATTAACAAATCACAC;  
3PV16 5' (C6, NH<sub>2</sub>) ACAACATTAGAACAGCAATACAACAAACCG;  
HPV18; 1PV18 5' (C6, NH<sub>2</sub>) TTAGAGAATTAAGACATTATTCAGACT;  
2PV18 5' (C6, NH<sub>2</sub>) CACCGCAGGCACCTTATTAATAAATTGTAT;  
25 3PV18 5' (C6, NH<sub>2</sub>) GACACATTGGAAAACTAACTAACACTGGG.

These oligonucleotides enable the PCR generation of various fragments; 3PV16 or 3PV18 with 2PV16 or 2PV18 respectively form a 62bp fragment; 1PV16 with 2PV16 form a 100bp fragment; 1PV18 with 2PV18 form a 103bp fragment. It will be appreciated that the 3PV16 and 3PV18 oligonucleotides can also be used as probes hybridizing to the products from the PCR reaction of 1PV16 with 2PV16 and 1PV18 with 2PV18, as well as hybridizing to the strand produced by the oligonucleotides 2PV16 and 2PV18 within the PCR. Oligonucleotides for the Ha-ras point mutation assays were as follows:

HRP1 5' (C6, NH2) GCGATGACGGAATATAAGCTGGTGGTGGTG;

HRP2 5' (C6, NH2) TTCTGGATCAGCTGGATGGTCAGCGCACTC;

These two oligonucleotide primers direct the PCR synthesis of an 80bp fragment. The sequences of the probes used for this point mutation study were as follows:

1T24 5' (C6, NH2) GGCGCCGTCGGTGTGGGCAA;

1CHR 5' (C6, NH2) GGCGCCGGCGGTGTGGGCAA;

2T24 5' (C6, NH2) TTGCCACACCGACGGCGCC;

2CHR 5' (C6, NH2) TTGCCACACCGCGGGCGCC.

Aside from these sequences we also synthesized the above 1CHR and 2T24 sequences without the 5' amino modification but with a 3' amino group. These 3' amino modified oligonucleotides were labeled with the ECL label and used in hybridizations. The site of the mutation/mismatch is indicated by the nucleotide in bold. The probes 1T24 and 1CHR hybridize to the strand produced by oligonucleotide HRP2 within the PCR. The probes 2T24 and 2CHR hybridize to the strand produced by oligonucleotide HRP1 within the PCR. Oligonucleotides JK8 and JK8C for coupling to particles:

JK8 5' (C6, NH2)GTCCAATCCATCTTGGCTTGTGGAAGTCTGA

JK8C 5' (C6, NH2)TCAGACTTCGACAACCCAAGATGGATTGGA1C.

These two sequences are derived from aequorin sequences and are complementary to each other.

JK7 5'TCAGACTTCGACAA(NH2)CCCAAGATGGATTGGA:

This oligonucleotide was amino modified using an amino modifier from Clontech (San Diego CA) which allows amino modifications within the sequence. JK7 was labeled using the Ru(bpy)<sub>3</sub><sup>2+</sup>-label.

Oligonucleotide probe for aequorin RNA generated by *in vitro* transcription:

T35 5' (NH2)GATTTTCCATTGTGGTTGACATCAAGGAA;

this oligo was labeled with both biotin and Ru(bpy)<sub>3</sub><sup>2+</sup>-label.

For the detection of Escherichia coli DNA we synthesized oligonucleotides specific for the Trp E/D region of the genome (3) as follows:

- TRP.CO3 5' (C6,NH2)GCCACGCAAGCGGGTGAGGAGTTCC(NH2);  
5 this sequence was labeled with Ru(bpy)<sub>3</sub><sup>2+</sup>-label and  
TRP.CO4 5' (C6,NH2)GTCCGAGGCAAATGCCAATAATGG  
was labeled with biotin as described below.

#### EXAMPLE 21

##### 10 Labeling Oligonucleotides

- All the synthetic oligonucleotides were purified to remove any contaminating amino groups by gel filtration on a Biogel P6 (BioRad Labs) column.  
15 Biotin was introduced via the 5'-amino group of the PCR primers using NHS-biotin (Clontech, San Diego CA) (4). Ru(bpy)<sub>3</sub><sup>2+</sup>-NHS was introduced via the amino group of the modified oligonucleotides as follows. The  
oligonucleotides (0.1μmole) in 100μl of PBS (pH 7.4)  
20 were reacted with 5μmole of Ru(bpy)<sub>3</sub><sup>2+</sup>-label dissolved in DMSO overnight at room temperature in the dark. Oligonucleotides were recovered from these labeling reactions by ethanol precipitation. Recent studies have demonstrated the ability to effectively label  
25 (>80%) using 0.5μmole of the Ru(bpy)<sub>3</sub><sup>2+</sup>-label (data not shown).

- The labeled oligonucleotides were further purified by HPLC on a reverse phase Vydac C-18 semiprep column with mobile phases of A) 100mM tetraethyl-  
30 ammonium acetate pH 7.0 and B) 50% A) and 50% acetonitrile, running the gradient from 20% to 40% of B.

- Probes 1CHR and 1T24 were also labeled with <sup>32</sup>P using T4 polynucleotide kinase using established  
35 methods generating probes with a specific activity of 77,000 cpm/ng (5).

EXAMPLE 22Preparation of Nucleic Acid Magnetic Particles

5 Dynal M 450 particles were activated with 2-fluoro-1-methylpyridinium toluene-4-sulfonate using standard procedures (6). These activated particles were then reacted with oligonucleotides JK8 and JK8C. To 100mg of activated Dynal particles were added 33 nmoles of oligonucleotide in 650  $\mu$ l of 0.1M NaHCO<sub>3</sub>,  
10 followed by incubation for 3 hours with mixing. The particles were blocked by the addition of ethanolamine (4mL, 0.1M). The coupled particles were mixed with 0.5mg/mL single stranded salmon sperm DNA in ECL buffer, washed 4-5 times into ECL buffer and  
15 resuspended at 10mg/mL in ECL buffer containing 100 $\mu$ g/mL single stranded salmon sperm DNA.

EXAMPLE 23Preparation of Streptavidin Magnetic Particles I

20 Dynal M 450 particles were activated with 2-fluoro-1-methylpyridinium toluene-4-sulfonate using standard procedures (6). The activated particles were then reacted with streptavidin (Sigma Ltd). Activated  
25 particles (50mg) were washed with 0.1M NaHCO<sub>3</sub> followed by the addition of streptavidin (1.5mg) and reacted overnight. The particles were blocked by the addition of ethanolamine (4mL, 0.1M). The coupled particles were mixed with 0.5mg/mL single stranded salmon sperm  
30 DNA in ECL buffer, washed 4-5 times into ECL buffer and resuspended at 10mg/mL in ECL buffer containing 100 $\mu$ g/mL single stranded salmon sperm DNA. The streptavidin particles from Dynal M-280 also proved valuable but gave lower signals with the current assay  
35 sequence. For immunoassay applications particles were

blocked with BSA after antigen or antibody coupling using the buffers used for passive coating.

EXAMPLE 24

5        Preparation of Streptavidin Magnetic Particles II

To 15 mg of BSA (in 2-3 mL PBS), 105  $\mu$ l of dimethylsulfoxide containing 50 mg/mL of biotin-x-NHS (Clontech, San Diego CA. 5002-1) was added followed by  
10 mixing and incubation at room temperature for 30 minutes. The reaction was stopped by adding 30  $\mu$ l of 1M glycine and incubation at room temperature for 10 minutes. The reaction mix was purified by gel filtration chromatography (Biorad, Bio-Gel P6). This  
15 biotin-BSA was filtered using 0.2  $\mu$ m syringe. 5 mg biotin-BSA in 10 mL of 0.2 M sodium carbonate/bicarbonate buffer pH 9.6 (carbonate/bicarbonate) buffer was added to 300 mg of Dynabeads washed with carbonate/bicarbonate (Dynal 14002). This mixture was  
20 Vortexed, and incubated overnight at room temperature with mixing. These particles were magnetically separated followed by the addition of 10 mL ECL diluent and 100  $\mu$ l tRNA (10 mg/mL). This mixture was incubated for 3-4 hours at room temperature with mixing. These  
25 particles were washed once with 10 mL of ECL diluent and resuspended in 10 mL of ECL diluent and 100  $\mu$ l tRNA (10 mg/mL). This mixture was mixed and incubated at 2-6 °C overnight to stabilize proteins on particles. The particles were magnetically separated and suspended in  
30 10 mL of PBS containing 15 mg of streptavidin (Scripps S1214) followed by mixing for one hour. The particles were washed 4 times in 10 mL ECL diluent, with 5 minutes mixing for each wash. The particles were finally resuspended in 29.7 mL of ECL diluent and 300  
35  $\mu$ l tRNA (10mg/mL) to a final concentration of 10 mg/mL particles + 100  $\mu$ g/mL tRNA.



EXAMPLE 25Detection of Immobilized DNA on Particles by Hybridization With ECL DNA Probes.

5      The ability to detect ECL after hybridization to particles was demonstrated by the hybridization of particles coupled to JK8 and JK8C (Example 22) with Ru(bpy)<sub>3</sub><sup>2+</sup>-label oligonucleotide JK7. Individual lots of particles (300 µg) in ECL buffer were mixed with 50µl of ECL buffer containing 12.5, 6.3, 3.01, and 1.5 fmoles of labeled JK7. These mixtures were hybridized for 4 hours at 52°C followed by washing with 1mL of ECL buffer and resuspension in 830µl of ECL buffer. These samples were analyzed as described in Example 1. The probe JK7 is complementary to the JK8 sequence and not complementary to JK8C sequence.

Table VIII

<u>Particles</u>	<u>Probe amount (fmoles)</u>	<u>ECL counts</u>
JK8	12.5	5085
	6.3	3035
	3.01	1345
	1.5	657
JK8C	12.5	451
	6.3	345
	3.01	256
	1.5	212

20      The results shown in Table VIII demonstrate the ability to detect by specific hybridization the presence of specific sequences directly immobilized on the surface of particles by ECL.

EXAMPLE 26RNA Assay Based on Bead Bound ECL

Dynal M450 particles were coated with  
5 antibody specific for RNA/DNA antibodies (7) following  
standard procedures (Example 10). Specific RNA species  
were generated using plasmids derived from our cloned  
aequorin gene (8). In brief, the plasmid pA5' was cut  
with EcoRI purified and subjected to *in vitro*  
10 transcription using T3 RNA polymerase generating T3-R1  
RNA (negative RNA). Also plasmid pA5' was cut with  
BamHI purified and subjected to *in vitro* transcription  
using T7 RNA polymerase generating T7-Bam RNA (positive  
RNA). These two RNA species thus represent two  
15 complementary RNA species. These RNA species were  
purified by extraction with an equal volume of  
phenol:chloroform (50:50) followed by chloroform  
extraction and precipitation of the supernatant using  
2.5 vols of ethanol. The amount of RNA was determined  
20 using gel electrophoresis and spectrophotometry. These  
methods are well established and known to those skilled  
in the art (9). Streptavidin was labeled with  
 $\text{Ru}(\text{bpy})_3^{2+}$ -label using established methods using a 25:1  
molar excess of  $\text{Ru}(\text{bpy})_3^{2+}$ -label over streptavidin  
25 (Example 13). This labeled streptavidin was purified  
using an iminobiotin column following established  
methods (10). The streptavidin was estimated to  
contain 10  $\text{Ru}(\text{bpy})_3^{2+}$ -labels per streptavidin tetramer.  
This labeled streptavidin was then complexed with  
30 biotinylated T35, this was achieved using a one to one  
mix of oligonucleotide to labeled streptavidin.  
Specifically 20pmoles of each were mixed in a final  
volume of 15 $\mu$ l of ECL buffer and incubated over night  
at 4°C to form the labeled streptavidin-  
35 oligonucleotide (SA-T35) complex. The samples of  
positive and negative RNA (10ng) were hybridized to 2 $\mu$ l

of the SA-T35 complex (one step assay) or 25ng of the biotinylated T35 (two step assay). Samples were made up to 50 $\mu$ l and hybridized for 3 hours at 50°C followed by the addition of 200 $\mu$ g of anti DNA/RNA antibody coated particles in 20 $\mu$ l of PBS 0.1%BSA. This mixture was mixed at room temperature for 1 hour followed by two washes in ECL buffer. Samples from the hybridization with the SA-T35 complex were resuspended in 530 $\mu$ l of ECL buffer and analyzed as described in Example 1. Those samples from the hybridization with biotinylated T35 alone were then incubated with 50pmoles of labeled streptavidin and incubated for 1hr with mixing followed by two washes in ECL buffer. Samples from the hybridization were resuspended in 530 $\mu$ l of ECL buffer and analyzed as described in Example 1. The results are presented in Table IX.

Table IX

<u>ASSAY</u>	<u>RNA</u>	<u>ECL COUNTS (average)</u>
One Step	Positive	815
	Negative	91
Two Step	Positive	1123
	Negative	194

20

EXAMPLE 27Polymerase Chain Reactions

Polymerase chain reactions were performed essentially as described (11, 12, 13). Reactions were typically of 100 $\mu$ l unless otherwise stated. PCR carried out in the asymmetric mode directed incorporation of the Ru(bpy)<sub>3</sub><sup>2+</sup>-label, using 5 pmoles of the biotinylated oligonucleotide and 50 pmoles of

Ru(bpy)<sub>3</sub><sup>2+</sup>-label oligonucleotide. We ran the assay for the Ha-ras point mutation under identical conditions but without the Ru(bpy)<sub>3</sub><sup>2+</sup>-labeled oligonucleotide.

Also, we ran the non-separation HPV assay

5 asymmetrically but making use of a ten fold excess of the biotinylated oligonucleotide typically 40 pmoles. The thermocycler conditions were as follows, for the direct incorporation HPV 18 and 16 assay, the profile was 93°C 1sec, 50°C 1sec, 60°C 2min; for the Ha-ras  
10 point mutation assay 93°C 1sec, 69°C 2min; for the non-separation HPV assay 93°C 10sec, 50°C 30sec, 60°C 2min. The cycle numbers for these PCR runs were from 30 to 40 depending on the assay and the required degree of sensitivity.

15

#### EXAMPLE 28

#### DNA-PROBE ASSAY FORMAT I. Detection and Quantitation of HumanPapilloma Virus PCR Products by Enzymatic Incorporation.

20

Following PCR using direct incorporation of the Ru(bpy)<sub>3</sub><sup>2+</sup>-label oligonucleotide, the whole reaction mixture (90-100μl) was added to 600μg of streptavidin coupled magnetic particles I, followed by incubation  
25 for 20min at room temperature with shaking. The solid phase in these samples was separated using magnetic racks, washed twice with ECL buffer, resuspended in 530μl of ECL buffer and then analyzed for electrochemiluminescence as described in Example 1.

30 Fig. 3 illustrates this assay format. The results for this assay format were demonstrated with human papilloma virus samples (2,14). Specificity studies of the direct incorporation of Ru(bpy)<sub>3</sub><sup>2+</sup>-label-oligonucleotides into biotinylated PCR products  
35 made use of the closely related virus types HPV16 and HPV18. Assay for the presence of HPV 16 and 18 was

made using DNA samples positive for both virus types and oligonucleotides specific for each virus type. The primers were as follows 2PV16, 2PV18 were biotinylated and 3PV16, 3PV18 were  $\text{Ru}(\text{bpy})_3^{2+}$ -label-oligonucleotides. The 2/3PV16 and 2/3PV18 oligonucleotides were specific for HPV 16 and 18 respectively. The resultant bead-captured  $\text{Ru}(\text{bpy})_3^{2+}$ -label was analyzed for ECL as described in Example 1. The results were plotted as ECL counts for each sample primer combination; see Fig. 6.

To demonstrated the quantitative nature of our assay format a standard curve of directly incorporated  $\text{Ru}(\text{bpy})_3^{2+}$ -label and biotinylated oligonucleotides into HPV16 PCR products was generated. The resultant bead-bound  $\text{Ru}(\text{bpy})_3^{2+}$ -label was analyzed for ECL as described in Example 1. The ECL peak photon counts were plotted verses increasing concentrations HPV 16 DNA, expressed as a ratio viral copies to total cellular DNA copies. The primers used in this analysis HPV16 were 1PV16 (biotin label) and 2PV16  $\text{Ru}(\text{bpy})_3^{2+}$ -label). DNA used for each PCR was maintained at a constant  $1\mu\text{g}$  using calf thymus DNA. The results for this standard curve are shown in Figure 7. These results of specificity and quantitation for this format demonstrates the ability of these ECL labels to produce simple and rapid DNA based assays. It also demonstrates the ability of the label to interface readily in enzyme reactions without interfering in the enzymatic process.

EXAMPLE 29DNA-PROBE ASSAY FORMAT II. Detection and Determination  
of Point Mutations in the Human Ha-ras Oncogene PCR  
Amplified Product

5

We carried out the PCR reactions of Ha-ras genes using oligos HRP1 and HRP2. Using biotinylated HRP1 with unlabeled HRP2 the resulting PCR product can hybridize to  $\text{Ru}(\text{bpy})_3^{2+}$ -label probes, 2CHR and 2T24. Conversely using biotinylated HRP2 with unlabeled HRP1 the resulting PCR product can hybridize to  $\text{Ru}(\text{bpy})_3^{2+}$ -label probes, 1CHR and 1T24. The DNA used was human placental (normal) and mouse NIH3T3 cell DNA, transfected with the mutant Ha-ras gene from the bladder carcinoma T24 (15).

15

The assay protocol was as follows; 90 $\mu\text{l}$  of PCR reaction mixture was added to 600 $\mu\text{g}$  of streptavidin coupled magnetic particles I, followed by incubation at room temperature for 30min. The solid phase in these samples was separated using magnetic racks, washed with 50mM NaOH, washed with hybridization buffer (0.9 M NaCl, 50mM NaPO<sub>4</sub>, pH 7.7, 5mM EDTA, 0.1% w/v ficoll, 0.1% w/v polyvinylpyrrolidone, 0.1% w/v bovine serum albumin) and resuspended in hybridization buffer containing 10 $\mu\text{g}/\text{mL}$  of the  $\text{Ru}(\text{bpy})_3^{2+}$ -label-oligonucleotide. These samples were hybridized for 15min at 66°C.

20

25

The solid phase was separated using magnetic racks, washed twice with 0.9M NaCl, 50mM NaPO<sub>4</sub>, pH 7.7, 5mM EDTA, washed with 3M tetramethylammonium chloride, 50mM Tris-HCl, pH 8.0, 2mM EDTA, 0.025% triton X-100 at room temperature once and at 66°C twice for 20min each. The solid phase was washed with ECL buffer three times, resuspended in 530 $\mu\text{l}$  ECL buffer and electrochemiluminescence detected as described in Example 1. Fig. 4 illustrates this assay format.

30

35

The assays for Ha-ras PCR products using  $p^{32}$  labeled probes were similar to those using  $Ru(bpy)_3^{2+}$ -label except the solid phase was finally resuspended in 250  $\mu$ l of ECL buffer. These suspended samples were then transferred to 5mL of scintillation fluid and counted on a Beckman LS-100C liquid scintillation counter.

In Fig. 8 we show data from a point mutation assay for the Ha-ras oncogene. The PCR was performed as illustrated in Fig. 4 using biotinylated HRP2 with unlabeled HRP1 (for probes 1T24 and 1CHR) and biotinylated HRP1 with unlabeled HRP2 (for probes 2T24 and 2CHR), generating bead-bound single stranded targets for hybridization. The DNA samples were the normal (Placenta) Ha-ras Gene and the mutant (NIH3T3-T24) Ha-ras Gene. The hybridization of the bead-bound DNA with  $Ru(bpy)_3^{2+}$ -label-1T24 (1T24),  $Ru(bpy)_3^{2+}$ -label-2T24 (2T24),  $Ru(bpy)_3^{2+}$ -label-1CHR (1CHR) and  $Ru(bpy)_3^{2+}$ -label-2CHR (2CHR) was followed by TEMAC washes. Resultant bead-bound  $Ru(bpy)_3^{2+}$ -label was analyzed for ECL as described in Example 1. Results were plotted as ECL counts for each sample probe combination. The results (Fig. 8) were as expected with the normal probes hybridizing well to the normal DNA (see the CHR probes) and the mutant probes hybridizing to the mutant gene (see the T24 probes). It was of interest that these probes did not all perform equivalently. To investigate this apparent anomaly we studied these probes further using  $p^{32}$  labeled probes with and without  $Ru(bpy)_3^{2+}$ -label. This Evaluation of the specificity of the  $Ru(bpy)_3^{2+}$ -label probes using  $p^{32}$  labeled probes for the Ha-ras oncogene was carried out as follows. The PCR was performed as described in Fig. 8 using only biotinylated HRP2 with unlabeled HRP1 (for probes 1T24 and 1CHR). The probes used were: 1T24 and 1CHR containing  $p^{32}$  (1T24-P, 1CHR-P) as controls; with the 1T24 and 1CHR containing both  $p^{32}$

and  $\text{Ru}(\text{bpy})_3^{2+}$ -label to determine the effects of the  $\text{Ru}(\text{bpy})_3^{2+}$ -label. The samples were washed as earlier with TEMAC. The resultant bead-bound  $\text{p}^{32}$  was analyzed on addition of scintillation cocktail in a

5 scintillation counter. The results were plotted as  $\text{p}^{32}$  counts per second for each sample probe combination (see Fig. 9). This result demonstrated that the  $\text{p}^{32}$  probes and the  $\text{Ru}(\text{bpy})_3^{2+}$ -labeled probes function equivalently and that problems with the probe

10 specificity are due to the specific probe sequences used. To further demonstrate this equivalence of our  $\text{Ru}(\text{bpy})_3^{2+}$ -label and  $\text{p}^{32}$  we conducted a comparison between these labeled probes. The amplification was performed as previously described using placental DNA,

15 using biotinylated HRP2 with unlabeled HRP1 (for probes 1T24 and 1CHR). The resultant PCR product was then sampled to give a set of samples containing differing amounts of product. These sets of samples were then hybridized with either probes labeled with  $\text{p}^{32}$  (1T24- $\text{p}^{32}$  and 1CHR- $\text{p}^{32}$ ) or  $\text{Ru}(\text{bpy})_3^{2+}$ -label (1T24- $\text{Ru}(\text{bpy})_3^{2+}$  and 1CHR- $\text{Ru}(\text{bpy})_3^{2+}$ ). The results from each study were then

20 normalized using the average value from each label for the  $90\mu\text{l}$  of sample. These normalized figures allow a more effective comparison of signal to background and the comparative response of the two methods. The inset

25 to Fig. 10 illustrates the response at the lower level of the dilution curve. The samples were handled as described earlier (Fig. 8 and Fig. 9). Results in Fig. 10 demonstrated the equivalency of the two labels with

30 indications of a better response from our  $\text{Ru}(\text{bpy})_3^{2+}$ -labeled probe. These studies demonstrated the ability of  $\text{Ru}(\text{bpy})_3^{2+}$ -labeled probes to function as well as  $\text{p}^{32}$  labeled probes in their ability to discriminate single base changes in sample DNA. This evidence indicates

35 that the  $\text{Ru}(\text{bpy})_3^{2+}$ -label does little to affect the properties of the labeled probe in hybridization reactions.



EXAMPLE 30DNA-PROBE ASSAY FORMAT III. Detection and Quantitation  
of Human Papilloma Virus PCR Products in a Non-  
Separation Assay.

5

For the non-separation assay on HPV 18, we performed an asymmetric PCR reaction with an excess of the biotinylated primer. This PCR reaction generates an excess of biotinylated single-stranded DNAs now  
10 available for direct hybridization by the  $\text{Ru}(\text{bpy})_3^{2+}$ -label-probes. For hybridization, we added 1000 ECL counts of  $\text{Ru}(\text{bpy})_3^{2+}$ -label-oligonucleotide (~ 2ng) specific for the HPV gene amplified to 15 $\mu\text{l}$  of the PCR after completion of the amplification followed by  
15 incubation for 15min at 50°C. To this hybridization mixture we added 60 $\mu\text{l}$  of ECL buffer containing 600 $\mu\text{g}$  of streptavidin coupled magnetic particles I and incubated with shaking at room temperature for 15min. The sample volume was increased to 530  $\mu\text{l}$  by addition of ECL  
20 buffer followed by detection of electrochemiluminescence as described in Example 1. Fig. 5 illustrates this assay format. To demonstrate this non-separation assay we ran a standard curve of HPV18 DNA. The PCR was performed using biotinylated  
25 2PV18 and unlabeled 1PV18 using HeLa DNA (14). The resultant PCR reaction was then hybridized with the specific probe  $\text{Ru}(\text{bpy})_3^{2+}$ -label- 3PV18. The hybridization mixture was then added to streptavidin coated particles and the resultant bead-bound  
30  $\text{Ru}(\text{bpy})_3^{2+}$ -label was directly analyzed for ECL as described in Example 1. The results were plotted as ECL counts verses HPV18 copies added to the PCR with a control of the ras oligonucleotide probe (see Fig. 11). These results demonstrate the ability to generate rapid  
35 non-separation assays for nucleic acid sequences based on the properties of the ECL assay system.

EXAMPLE 31Assay for Specific Genomic DNA Sequences.

The assay format described here makes use of two oligonucleotides, both of which hybridize to the same DNA strand next to each other, one probe allows capture; the other labels the complex (sandwich hybridization). This assay was demonstrated using E.coli DNA and probes specific for the trp E/D gene region. The E.coli DNA was prepared following standard protocols (16). The salmon sperm control DNA was purchased from Sigma Ltd. To the samples of DNA were added 14 $\mu$ l of hybridization buffer (10X PBS, 10mMEDTA and 0.7%SDS), 2ng of biotin labeled TRP.CO4 and 5ng of Ru(bpy)<sub>3</sub><sup>2+</sup>-label TRP.CO3. These samples were made up to 100 $\mu$ l with water. The samples were heated to 97°C and incubated at 97°C for 10min, cooled to 50°C and hybridized for 2hrs. To these samples we added 20 $\mu$ l of streptavidin coated magnetic particles II and mixed for 2hrs at room temperature. The particles were then washed 4 times in ECL buffer resuspended in 500 $\mu$ l of ECL buffer and analyzed as described in Example 3. The positive DNA is E.coli and the negative DNA is salmon sperm. The results are shown in Table X.

**Table X**

<u>DNA</u>	<u>Amount</u>	<u>Average ECL counts</u>
Positive	10	184
	25	257
	50	266.5
Negative	10	87
	25	70
	50	75

25

These results demonstrated the ability of the ECL assay system to function in the detection of a genomic gene

in E.coli using a sandwich hybridization assay format on non amplified DNA. The streptavidin coated magnetic particles I can be similarly used in the fashion that the streptavidin coated magnetic particles II are used in this example.

### EXAMPLE 32

#### Particle Concentration on Evanescent-Wave Fluorescence Detectors

Concentration of labeled complex on a detection surface can be used to increase sensitivity of assays using evanescent wave detectors. Such detectors may use either optical fiber(s) or planar optical waveguide(s) 300 to carry light 310 from a light source to the fluid environment. The light is reflected through the waveguide or optical fiber by total internal reflection (TIR) 310' which occurs when an incident light beam strikes an interface between a dielectric medium of high refractive index ( $n_1$ ) and one of lower refractive index ( $n_2$ ). When the incident angle of the light beam is greater than the critical angle 315 which is  $\theta_0$  (the angle shown between perpendicular line 300' and the path of light 310'),  $\theta_0 = \sin^{-1}(n_2/n_1)$ , then the light is 100% internally reflected at the interface. In optical waveguides and optical fibers, light travels with an incidence angle greater than this critical angle, and propagates through the medium by total internal reflectance. Fig. 22 depicts the TIR propagation in a waveguide or optical fiber.

Although the light ray is totally reflected at each interaction with the interface, the electromagnetic field is not zero outside the medium. Physical requirements of continuity across an interface require that the electromagnetic field decay exponentially as it penetrates outside the fiber or

waveguide into the external environment. This field is called the evanescent field 320 and is capable of exciting fluorophores to fluoresce. The decay rate of the evanescent field depends on the incident wavelength, refractive indices  $n_1$  and  $n_2$ , and the angle of incidence. Using a quartz waveguide and visible light in a water environment, the evanescent field 320 decays by approximately 90% within a distance of 100 nm from the waveguide/solution interface. In Fig. 22 surrounding medium 330 has a refractive index  $n_2$  and the optical fiber(s) or waveguide(s) 300, a refractive index  $n_1$ .

The same principles which create the evanescent field for light propagating in the waveguide or optical fiber allow the light which is generated when fluorophores luminesce to be captured back into the optical element efficiently. Additionally, any light produced outside the evanescent zone (320) is efficiently rejected from entering the optical element. The combination of these effects allows optical fibers or waveguides to be used as efficient optical elements for measuring the presence of and concentration of fluorophore labels on or near their surfaces in an aqueous environment. U.S. Patent No. 4,447,546, incorporated herein by reference, describes one suitable method and apparatus for conducting fluorescence immunoassays employing an optical fiber to excite and measure evanescent zone fluorescence from labelled immunoreactant.

The invention can be applied to improve the sensitivity of fluorescent binding assays using optical fibers or waveguides. The assay is performed using reagents labelled with fluorescent moieties. After incubation of the particles, sample and reagents, the particles are concentrated upon the surface of the waveguide or optical fiber. Because the surface area

of the particles is greater than the geometric area of the waveguide or optical fiber, more fluorophores can be collected in the evanescent zone surrounding the optical element. Hence, the luminescent signal from the particles will be larger and quantitation of analyte will be more sensitive, resulting in improved detection limits.

Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited by particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope of the present invention.

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WHAT IS CLAIMED IS:

1. A method for performing a binding assay for an analyte of interest present in a sample comprising the steps of:

- (a) forming a composition containing
  - (i) said sample
  - (ii) an assay-performance-substance which contains a component linked to a label compound capable of being induced to luminesce, and
  - (iii) a plurality of particles capable of specifically binding with the analyte and/or said assay-performance-substance;
- (b) incubating said composition to form a complex which includes a particle and said label compound;
- (c) collecting said complex in a measurement zone;
- (d) inducing the label compound in said complex to luminesce by surface selective excitation, and
- (e) measuring the emitted luminescence to measure the presence of the analyte of interest in the sample.

2. A method as recited in claim 1 wherein said complex is collected on the surface of means for inducing luminescence and measuring said luminescence.

3. A method as recited in claim 2 based upon measurement of electrochemiluminescence wherein said complex is collected at an electrode surface.

4. A method as recited in claim 2 based on Raman excitation.

5. A method as recited in claim 2 based on total-internal-reflection-fluorescence.

6. A method as recited in claim 2 based upon surface plasmon resonance.

7. A method for performing a binding assay for an analyte of interest present in a sample based upon measurement of electrochemiluminescence at an electrode surface comprising the steps:

- (a) forming a composition containing
  - (i) said sample
  - (ii) an assay-performance-substance which contains a component linked to a label compound capable of being induced to electrochemiluminesce, and
  - (iii) a plurality of particles capable of specifically binding with the analyte and or said assay-performance-substance;
- (b) incubating said composition to form a complex which includes a particle and said label compound;
- (c) collecting said complex;
- (d) causing said collected complex to come in contact with an electrode surface and inducing the label compound in said complex to luminesce by impressing a voltage on said electrode; and
- (e) measuring the emitted luminescence at the electrode surface to measure the presence of the analyte of interest in the sample

8. A method for performing a binding assay for an analyte of interest present in a sample based upon measurement of electrochemiluminescence at an electrode surface comprising the steps:

- (a) forming a composition containing
  - (i) said sample

- (ii) an assay-performance-substance which contains a component linked to a label compound capable of being induced to electrochemiluminesce, and
  - (iii) a plurality of suspended particles having a density greater than the balance of said composition and being capable of specifically binding with the analyte and or said assay-performance-substance;
- (b) incubating said composition to form a complex which includes a particle and said label compound;
- (c) introducing said composition into an assay cell;
- (d) collecting said complex at the surface of an electrode located below at least a substantial portion of the volume of said assay cell by permitting said composition to reside in said cell for a time sufficient to permit the particles to settle upon said electrode surface by the force of gravity;
- (e) inducing the label compound in said collected complex to luminescence by imposing a voltage on said electrode; and
- (f) measuring the emitted luminescence at the electrode surface to measure the presence of the analyte of interest in the sample

9. A method as recited in claim 8 conducted as a batch process, the composition being permitted to reside within said cell for a time sufficient to permit settling of said particles upon said electrode surface.

10. A method as recited in claim 8 conducted as a flow process wherein said composition is flowed through said cell at a sufficiently low rate to permit

settling of at least a portion of said particles upon said electrode surface.

11. An assay method as recited in claim 8 wherein said particles have a density of from 0.1 to 5g/mL.

12. An assay method as recited in claim 11 wherein said particles have a density of from 0.5 to 2g/mL.

13. An assay method as recited in claim 8 wherein the size of said particles, measured as the mean diameter, ranges from 0.01 to 100  $\mu\text{m}$ .

14. An assay method as recited in claim 13 wherein the size of said particles ranges from 0.01 to 10  $\mu\text{m}$

15. An assay method as recited in claim 8 wherein the concentration of particles in said composition is from 1 to 10,000  $\mu\text{g/mL}$ .

16. An assay method as recited in claim 15 wherein said concentration of particles is in the range of from 5 to 1000  $\mu\text{g/mL}$ .

17. An assay method as recited in claim 8 wherein the density, size and concentration of said particles in said composition is such that the settling rate is at least 0.5 mm/minute.

18. An assay method as recited in claim 8 wherein at least a substantial portion of said electrode surface is covered by a monolayer of said complex prior to inducing electrochemiluminescence.

19. A method for performing a binding assay for an analyte of interest present in a sample based upon measurement of electrochemiluminescence at an electrode surface comprising the steps:

- (a) forming a composition containing
  - (i) said sample
  - (ii) an assay-performance-substance which contains a component linked to a label compound capable of being induced to electrochemiluminesce, and
  - (iii) a plurality of suspended particles having a density greater than the balance of said composition and being capable of specifically binding with the analyte and or said assay-performance-substance;
- (b) incubating said composition to form a complex which includes a particle and said label compound;
- (c) collecting said complex by centrifugation;
- (d) causing said collected complex to come in contact with an electrode surface and inducing the label compound in said complex to luminescence by imposing a voltage on said electrode; and
- (e) measuring the emitted luminescence at the electrode surface to measure the presence of the analyte of interest in the sample.

20. A method as recited in claim 19 wherein said centrifugation step collects said complex at the surface of said electrode.

21. An assay method as recited in claim 19 wherein said particles have a density of from 0.1 to 5g/mL.

22. An assay method as recited in claim 21 wherein said particles have a density of from 0.5 to 2g/mL.

23. An assay methods is recited in claim 19 wherein luminescence is measured while said sample is being centrifuged.

24. A method for performing a binding assay for an analyte of interest present in a sample based upon measurement of electrochemiluminescence at an electrode surface comprising the steps:

- (a) forming a composition containing
  - (i) said sample
  - (ii) an assay-performance-substance which contains a component linked to a label compound capable of being induced to electrochemiluminesce, and
  - (iii) a plurality of suspended particles capable of specifically binding with the analyte and or said assay-performance-substance;
- (b) incubating said composition to form a complex which includes a particle and said label compound;
- (c) collecting said complex by filtration;
- (d) causing said collected complex to come in contact with an electrode surface and inducing the label compound in said complex to luminescence by imposing a voltage on said electrode; and
- (e) measuring the emitted luminescence at the electrode surface to measure the presence of the analyte of interest in the sample.

25. A method as recited in claim 24 wherein said filtration step collects said complex at the surface of said electrode.

26. An assay method as recited in claim 24 wherein the size of said particles, measured as the mean diameter, is from 0.001 to 100  $\mu\text{m}$

27. An assay method as recited in claim 26 wherein the size of said particles ranges from 0.01 to 10  $\mu\text{m}$ .

28. A method as recited in claim 24 wherein said filtration takes place on a porous metallic electrode surface wherein said pore size, measured as mean diameter, is from 10 to 90% of the mean diameter of said particles.

29. A method for performing a binding assay for an analyte of interest present in a sample based upon measurement of electrochemiluminescence at an electrode surface comprising the steps:

- (a) forming a composition containing
  - (i) said sample
  - (ii) an assay-performance-substance which contains a component linked to a label compound capable of being induced to electrochemiluminesce, and
  - (iii) a plurality of magnetically responsive suspended particles capable of specific binding with the analyte and or said assay-performance-substance;
- (b) incubating said composition to form a complex which includes a particle and said label compound;
- (c) collecting said complex by imposition of a magnetic field on said particles;
- (d) causing said collected complex to come in contact with an electrode surface and inducing the label compound in said complex to luminescence by imposing a voltage on said electrode; and

(e) measuring the emitted luminescence at the electrode surface to measure the presence of the analyte of interest in the sample.

30. A method as recited in claim 19 wherein the imposition of said magnetic field causes said complex to collect at the surface of said electrode.

31. A method for performing a binding assay for an analyte of interest present in a sample based upon measurement of electrochemiluminescence at an electrode surface comprising the steps:

- (a) forming a composition containing
  - (i) said sample
  - (ii) an assay-performance-substance which contains a component linked to a label compound capable of being induced to electrochemiluminesce, and
  - (iii) a plurality of magnetically responsive suspended particles capable of specific binding with the analyte and or said assay-performance-substance;
- (b) incubating said composition to form a complex which includes a particle and said label compound;
- (c) introducing said composition into an assay cell;
- (d) collecting said complex at the surface of an electrode by imposition of a magnetic field on said particles;
- (e) inducing the label compound in said collected complex to luminescence by imposing a voltage on said electrode; and
- (f) measuring the emitted luminescence at the electrode surface to measure the presence of the analyte of interest in the sample.



32. A method as recited in claim 31 conducted as a batch process, the composition being permitted to reside within said cell for a time sufficient to permit said magnetic field to cause said particles to settle upon said electrode surface.

33. A method as recited in claim 31 conducted as a flow process wherein said composition is flowed through said cell at a sufficiently low rate to permit said magnetic field to cause said particles to settle upon said electrode surface.

34. An assay method as recited in claim 31 wherein said particles have a magnetic susceptibility of at least 0.001 cgs units.

35. A method as recited in claim 34 wherein the magnetic susceptibility is at least 0.01 cgs units.

36. An assay method as recited in claim 31 wherein said particles have a density of from 0.5 to 2 g/mL.

37. A method as recited in claim 36 wherein the density of said particles is from 0.5 to 2 g/mL.

38. An assay method as recited in claim 31 wherein the size of said particles, measured as the mean diameter, is from 0.001 to 100  $\mu\text{m}$ .

39. An assay method as recited in claim 31 wherein the size of said particles is from 0.01 to 10  $\mu\text{m}$  and the particles have low resonance.

40. An assay method as recited in claim 31 wherein the concentration of particles in said composition is from 1 to 10,000  $\mu\text{g/mL}$ .

41. An assay method as recited in claim 40 wherein said concentration of particles is from 5 to 1000  $\mu\text{g/mL}$ .

42. An assay method as recited in claim 31 wherein the magnetic susceptibility, density, size and concentration of said particles in said composition is such that the settling rate of said particles is at least 0.5 mm/min.

43. An assay method as recited in claim 31 wherein at least a substantial portion of said electrode surface is covered by a monolayer of said complex prior to inducing electrochemiluminescence.

44. A method as recited in claim 31 wherein the lines of force of said magnetic field are substantially parallel with the surface of said electrode in the region of said electrode surface.

45. A method as recited in claim 31 wherein the magnetic field is withdrawn after collection of said complex and before inducing luminescence.

46. An apparatus for performing a binding assay for an analyte of interest present in a sample based upon measurement of electrochemiluminescence at an electrode surface comprising:

- (a) a cell defining a sample containing volume having a vertical columnar zone and having inlet and outlet means, and further including a substantially horizontally oriented

- electrode positioned below a substantial volume of said cell and said columnar zone;
- (b) means to impress a voltage upon said electrode; and
  - (c) means to measure the electrochemiluminescence generated at said electrode.

47. An apparatus for performing a binding assay for an analyte of interest present in a sample based upon measurement of electrochemiluminescence at an electrode surface comprising:

- (a) a cell defining an assay sample containing volume and having inlet and outlet means, an electrode, and further including means for obtaining a substantially even distribution of said complex on said electrode surface;
- (b) means to impress a voltage upon said electrode; and
- (c) means to measure the electrochemiluminescence generated at said electrode.

48. An apparatus as defined in claim 47 wherein said means for obtaining an even distribution of complex includes means for generating a magnetic field oriented with respect to said electrode so that the lines of force of said magnetic field are substantially parallel with the surface of said electrode in the region of said surface.

49. An apparatus as recited in claim 48 wherein said means for generating a magnetic field includes a magnet in north-south orientation positioned vertically below said electrode.

50. A composition of matter for use as a reagent in a microparticulate-based binding assay

comprising particles and at least one other component selected from the group consisting of:

- (a) electrolyte;
- (b) label compound containing an ECL moiety;
- (c) analyte of interest or an analog of the analyte of interest;
- (d) a binding partner of the analyte of interest or of its analog;
- (e) a reactive component capable of reacting with (c) or (d);
- (f) a reductant; and
- (g) an electrochemiluminescent-reaction enhancer, provided, however, that no two components contained within any reagent composition are reactive with one another during storage so as to impair their function in the intended assay.

51. A reagent as recited in claim 50 containing magnetically responsive particles.

52. An assay reagent for an assay based upon a binding reaction and the measurement of an electrochemiluminescent phenomenon comprising:

- (a) an electrolyte;
- (b) a plurality of magnetically responsive particles having a surface capable of binding to a component of the assay composition; and
- (c) a label substance having binding properties, said label substance including a chemical moiety having electrochemiluminescent properties.

53. A kit containing reagents for use in a microparticulate-based binding assay comprising:

- (a) magnetically responsive particles and at least one other single reagent selected from the group consisting of:
  - (i) electrolyte;
  - (ii) label compound containing an ECL moiety;
  - (iii) analyte of interest or an analog of the analyte of interest;
  - (iv) a binding partner of the analyte of interest or of its analog;
  - (v) a reactive component capable of reacting with (c) or (d);
  - (vi) a reductant; and
  - (vii) an electrochemiluminescent-reaction enhancer, or
- (b) one or more reagents, at least one of which contains magnetically responsive particles, said reagents containing components selected from the aforesaid components (i)-(vii);  
provided, however, that no two or more components of such mixed reagents react with one another under storage conditions so as to impair the function of the reagents in the intended assay.

1 / 16

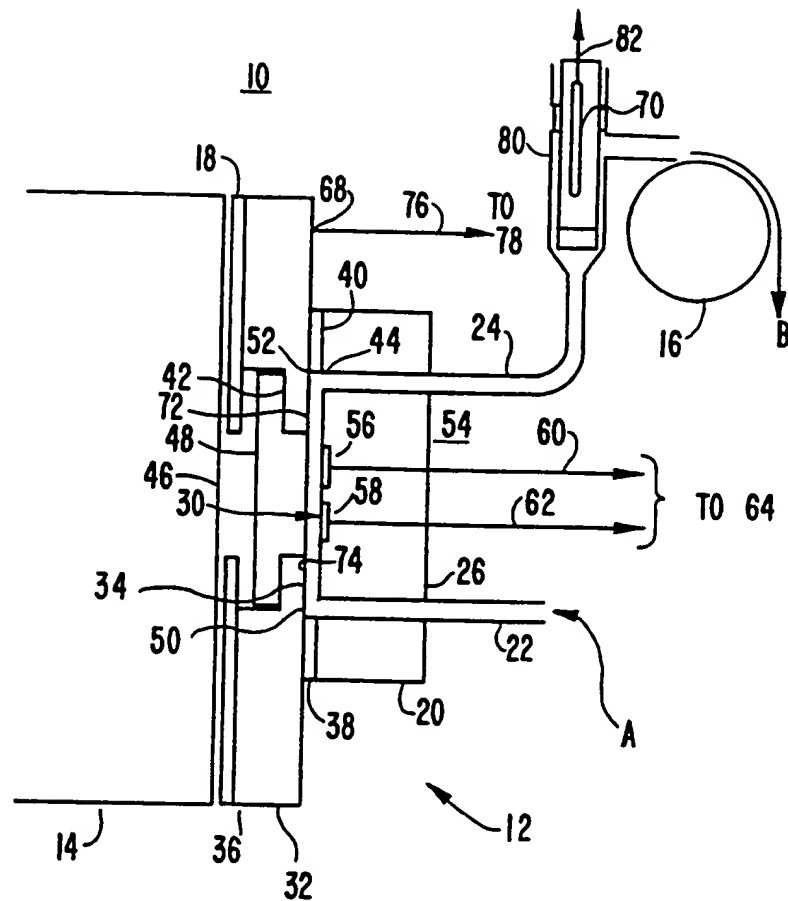


FIG. 1

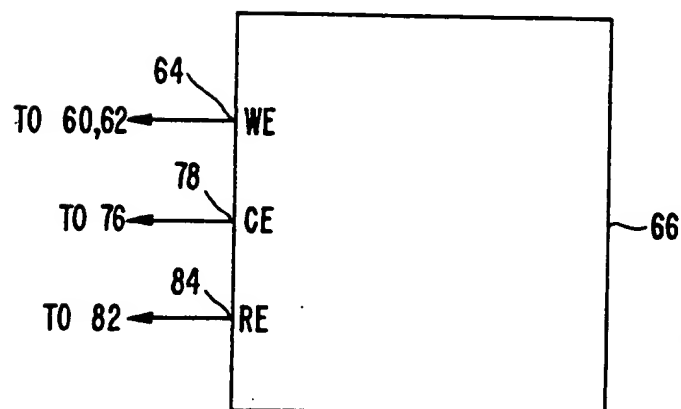


FIG. 2

SUBSTITUTE SHEET

2 / 16

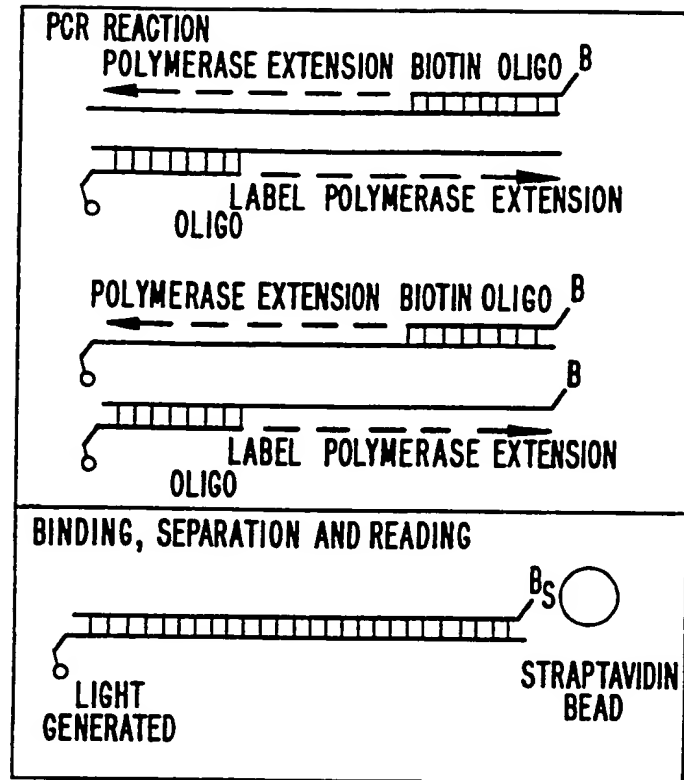


FIG. 3

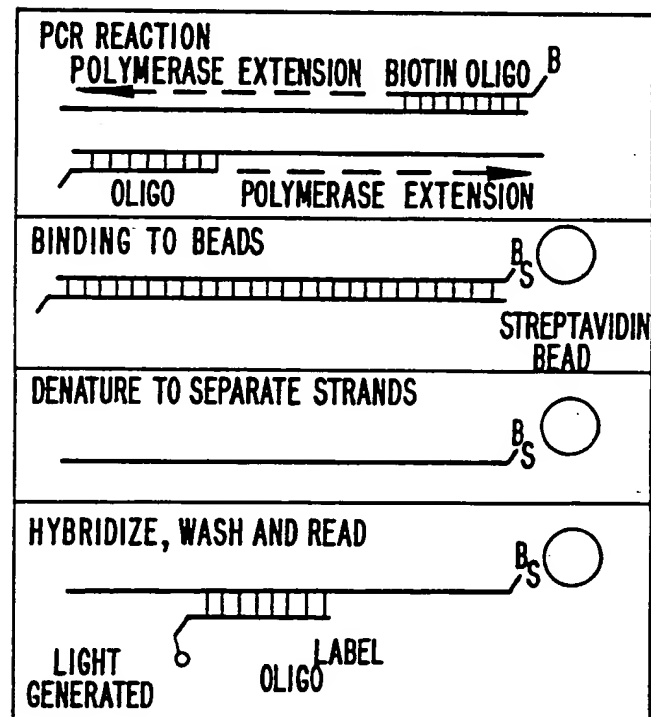


FIG. 4

3 / 16

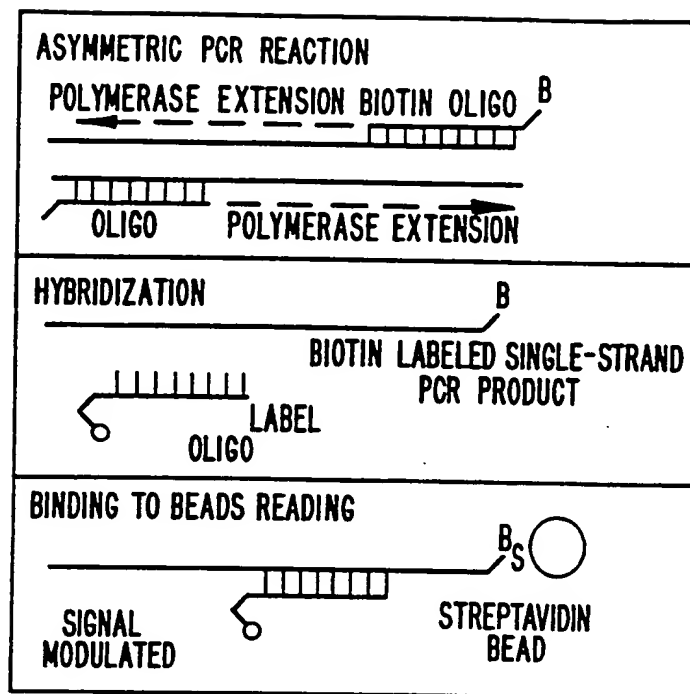


FIG. 5

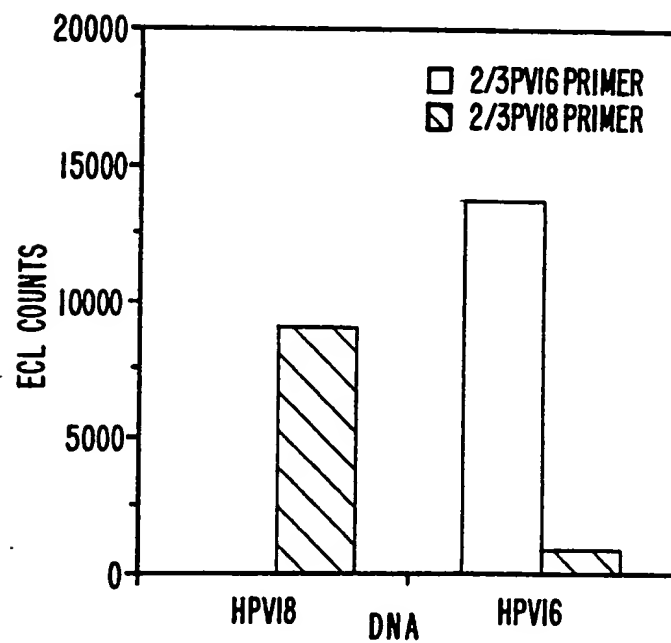


FIG. 6

SUBSTITUTE SHEET



4 / 16

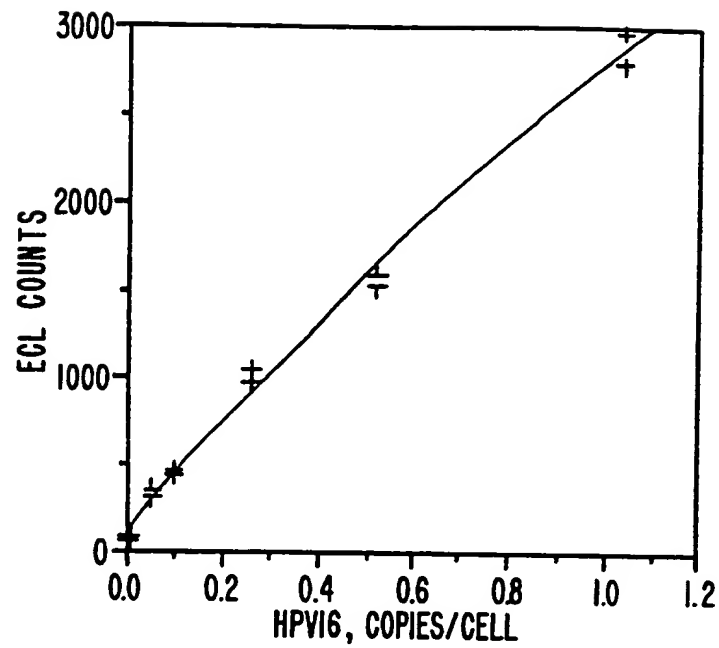


FIG. 7

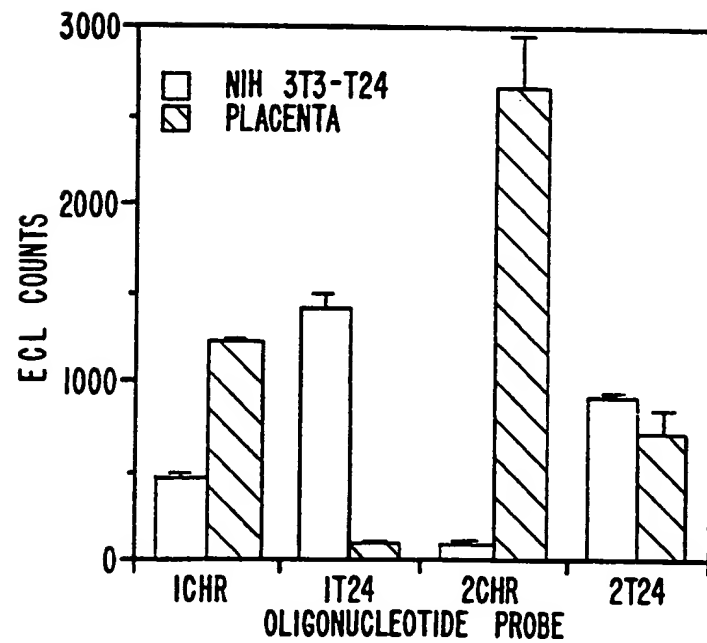


FIG. 8

5 / 16

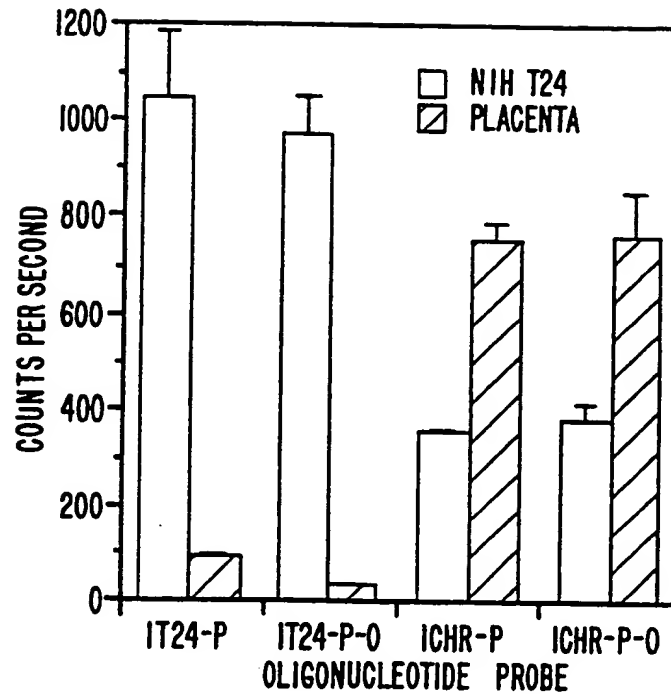


FIG. 9

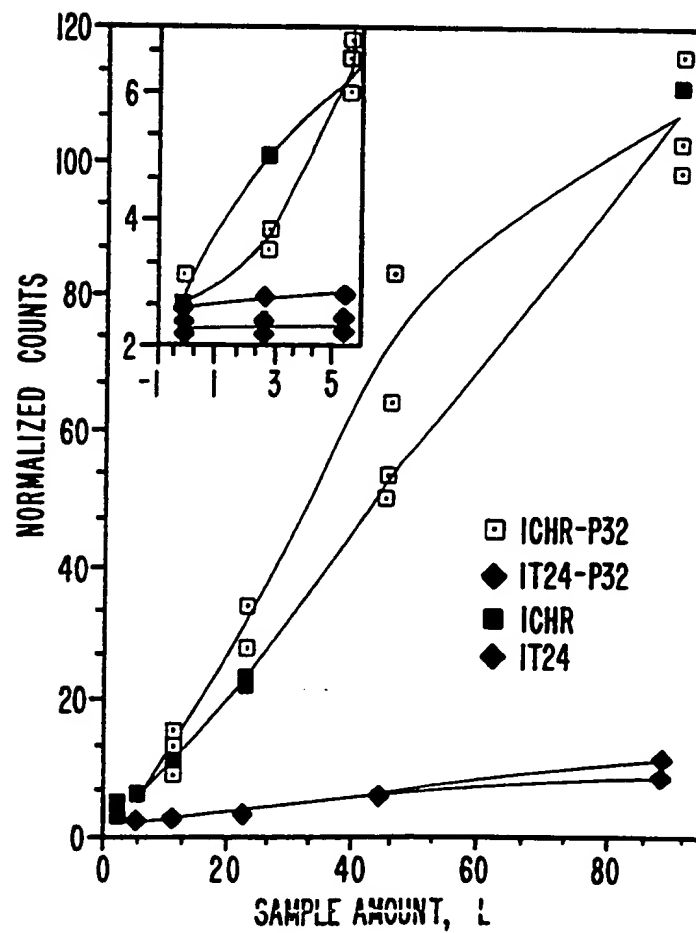


FIG. 10

6 / 16

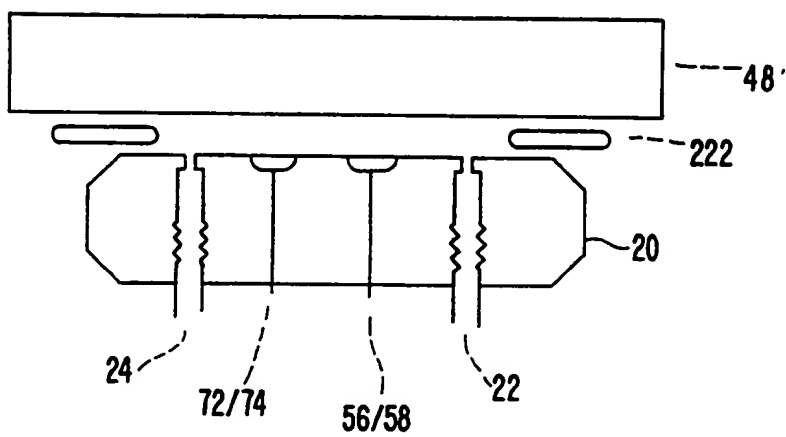
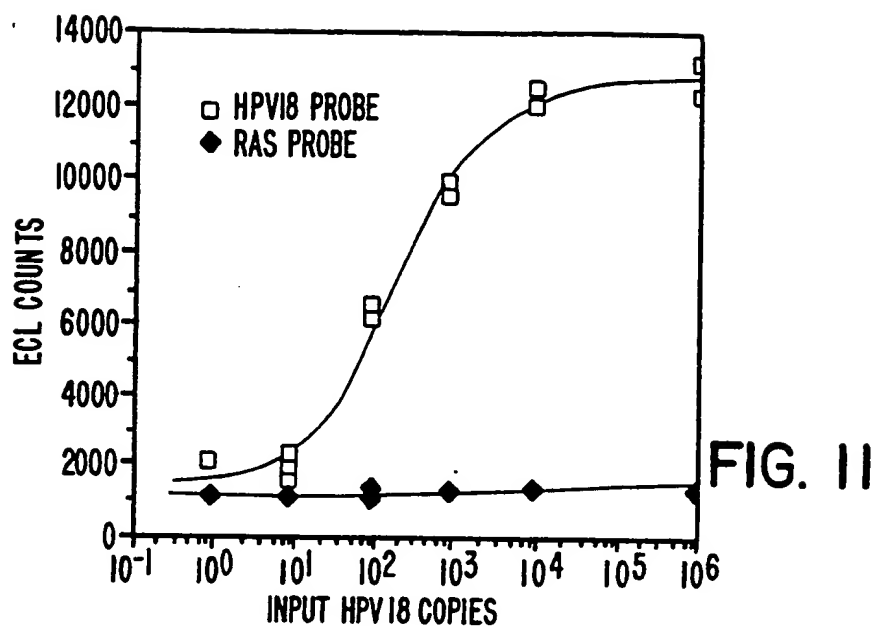


FIG. 12

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7/ 16

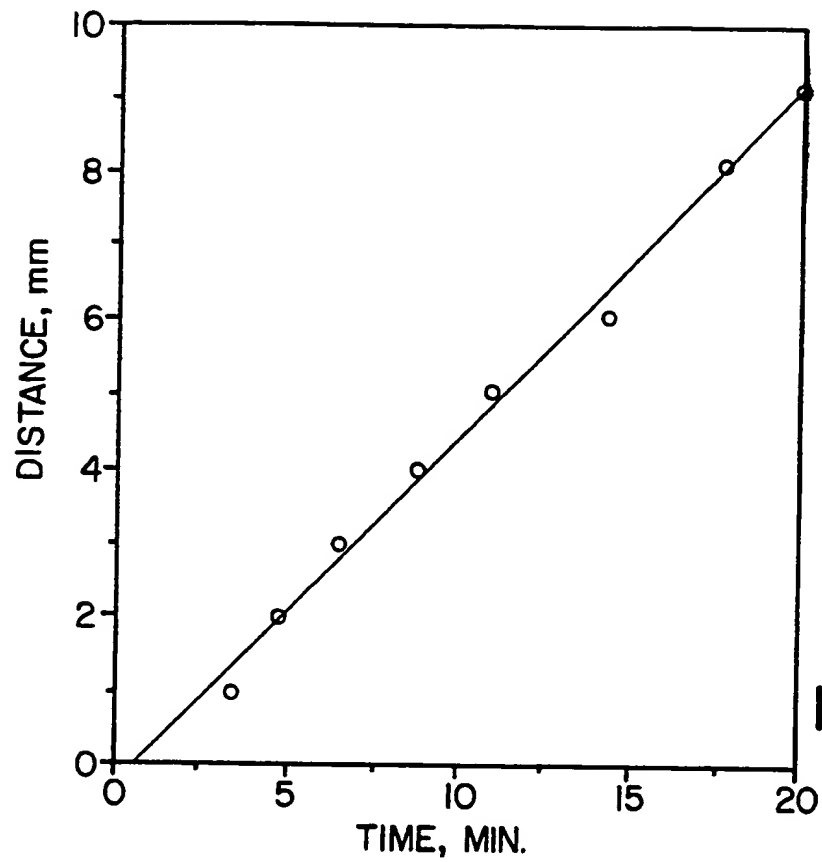


FIG. 13

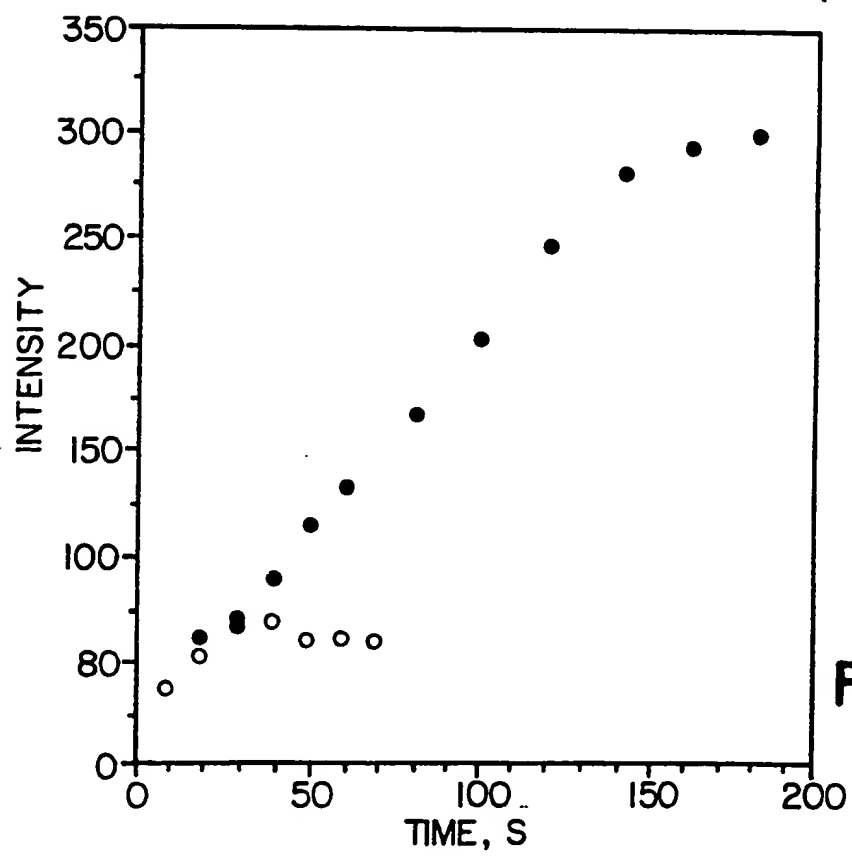


FIG. 14

8 / 16

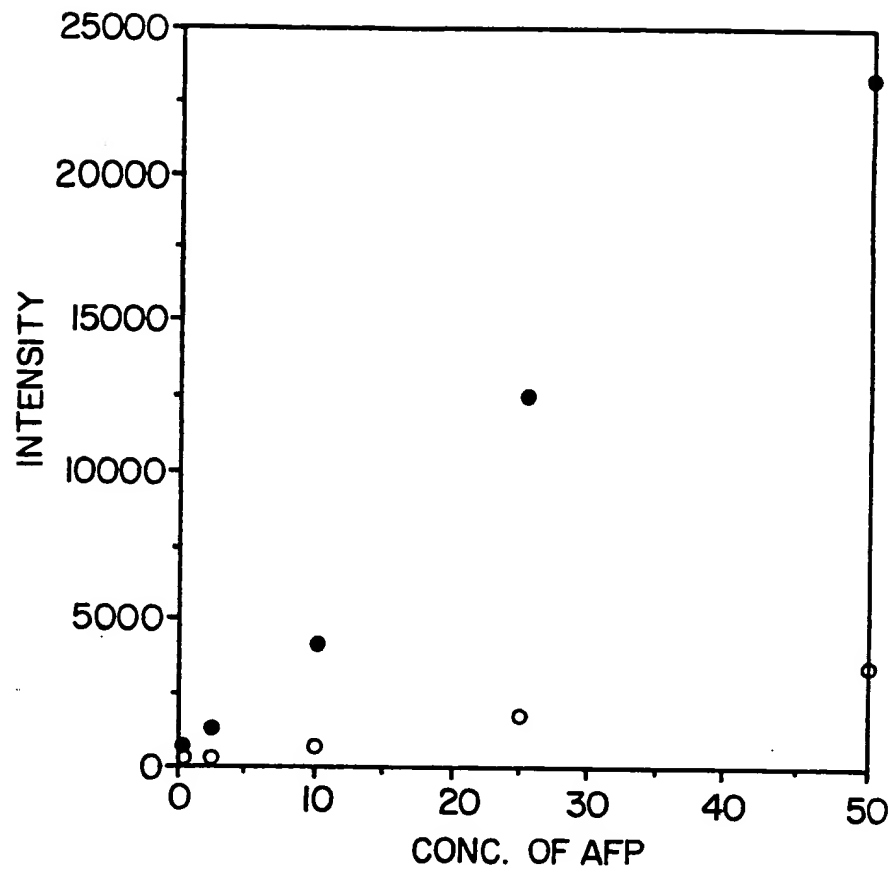


FIG. 15

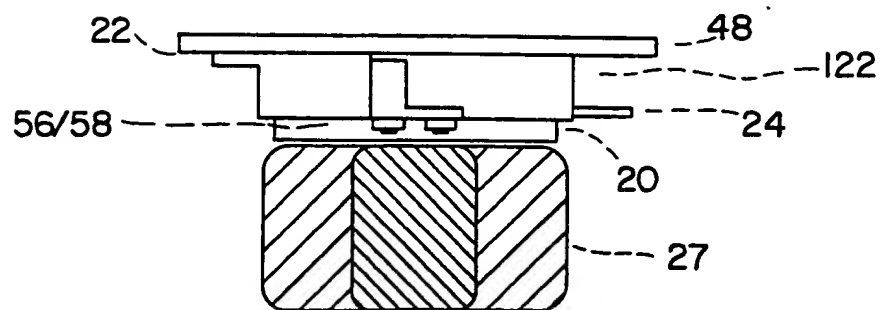


FIG. 16

9 / 16

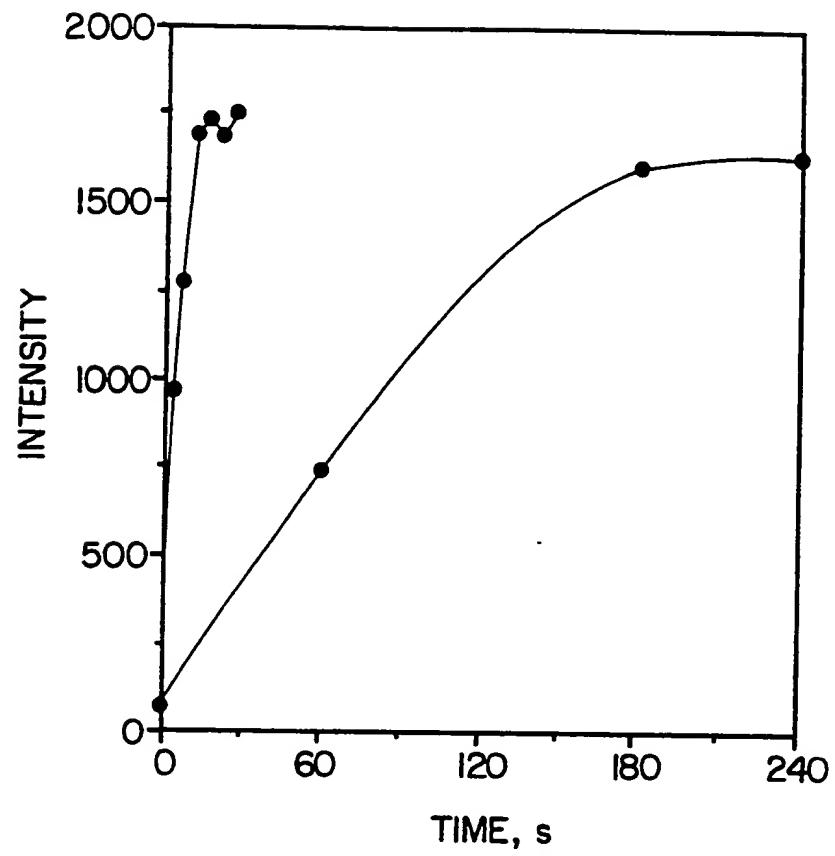


FIG. 17

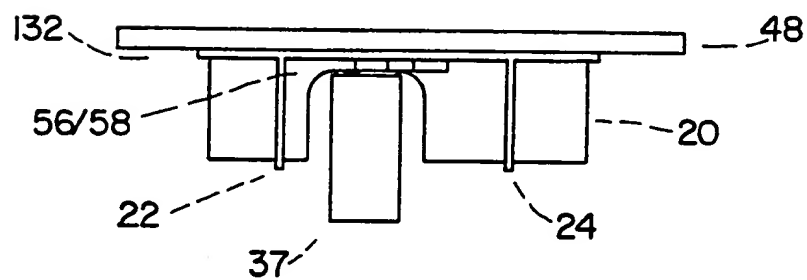


FIG. 18

10/16

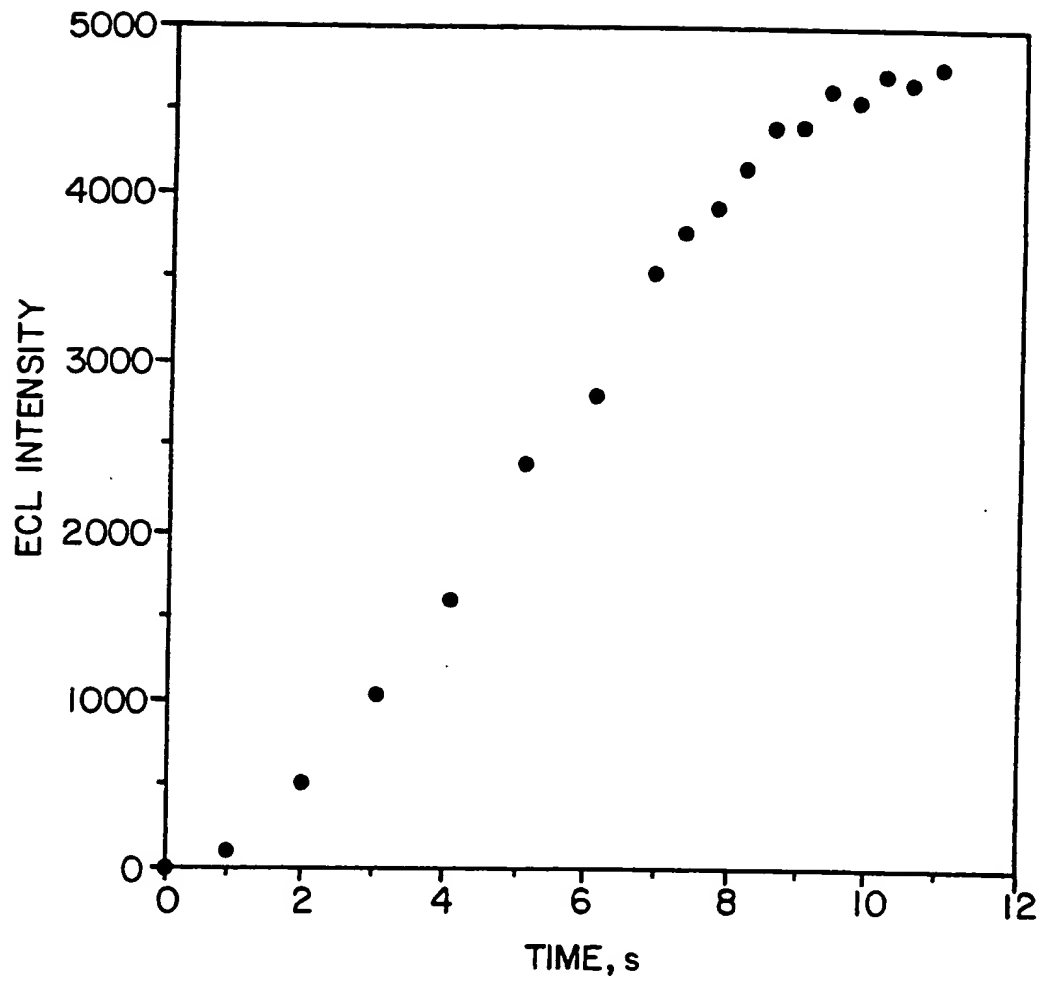


FIG. 19

11 / 16

FIG. 20A

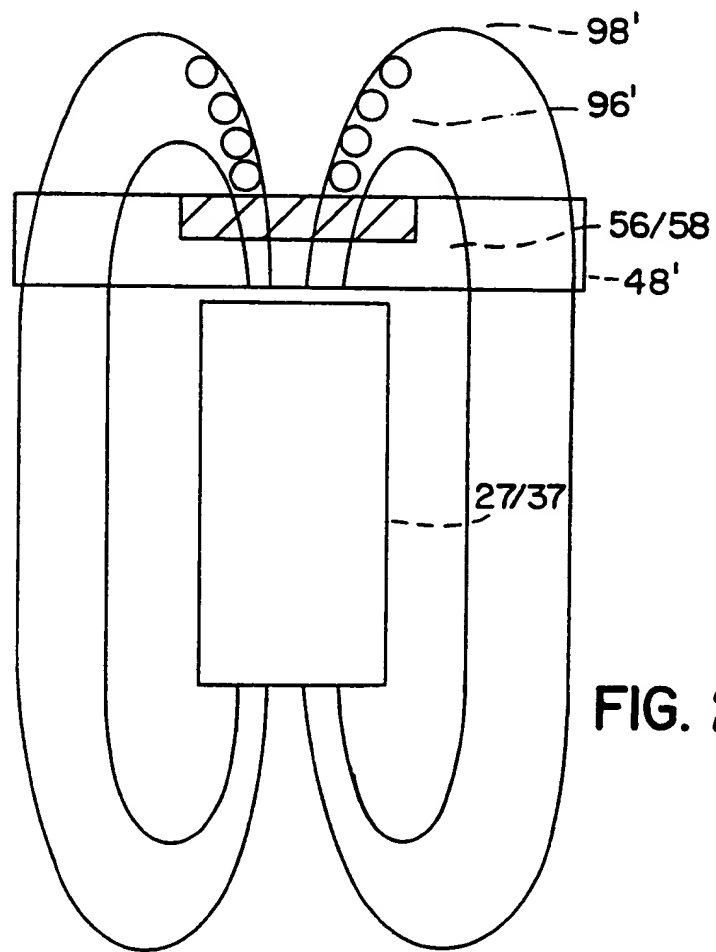
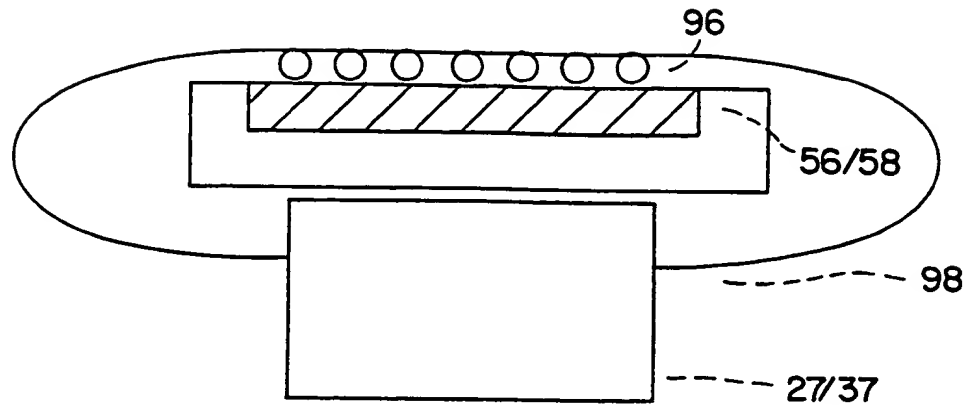


FIG. 20B



12 / 16

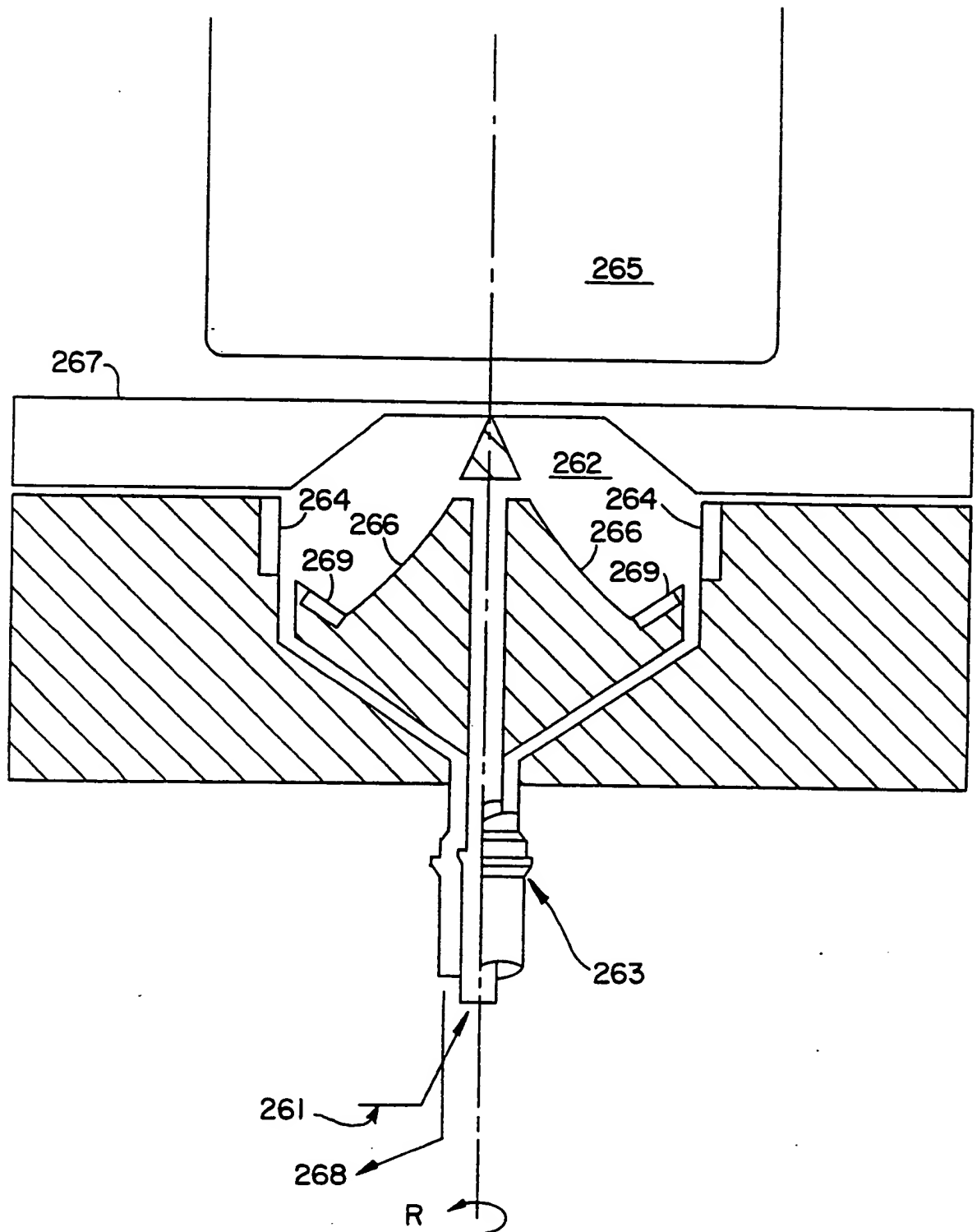


FIG. 21

13 / 16

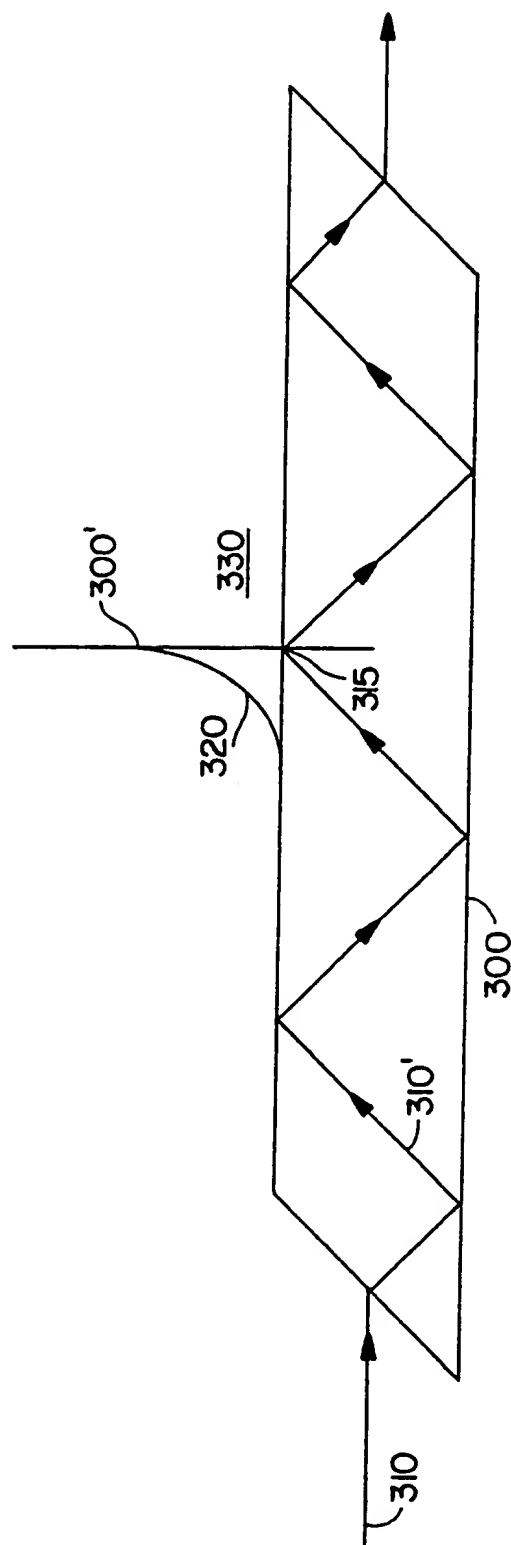
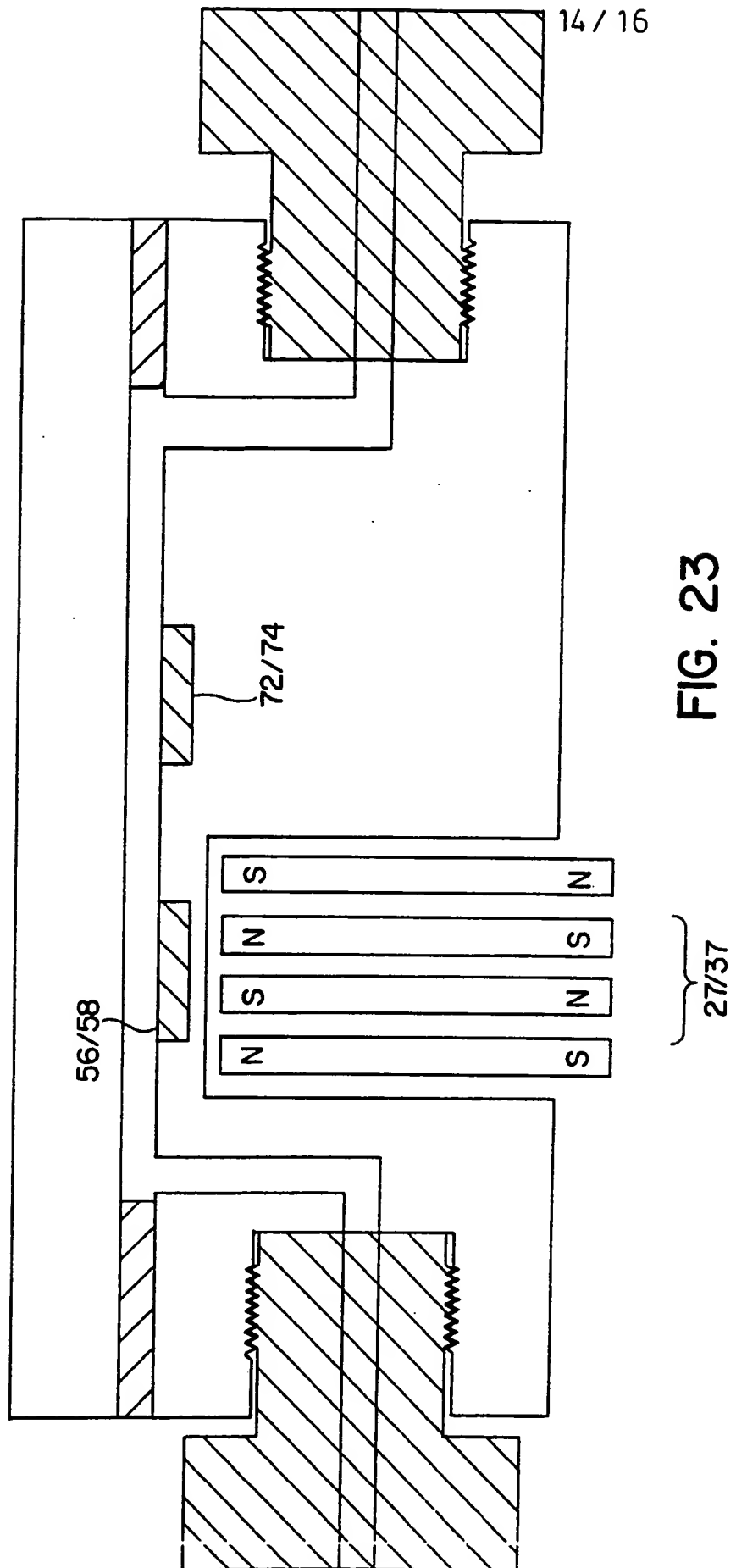


FIG. 22



15/16

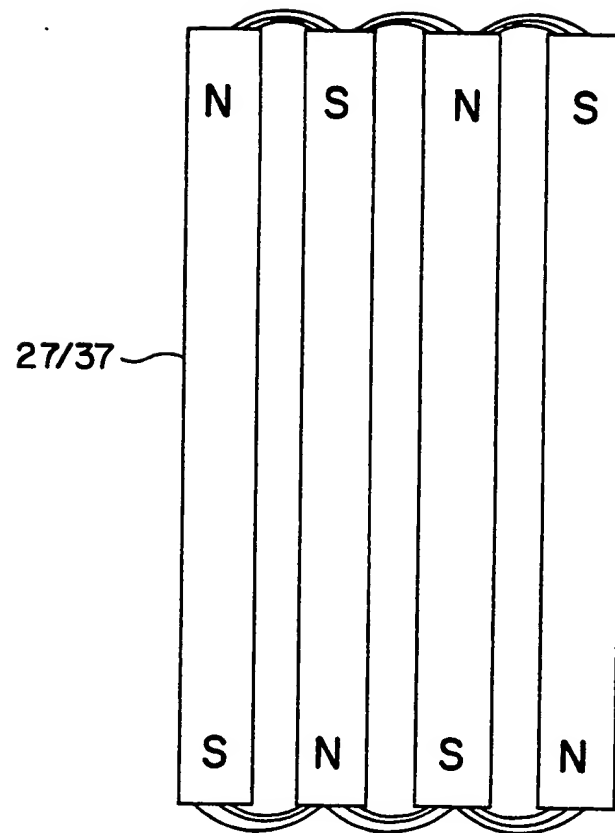


FIG. 24

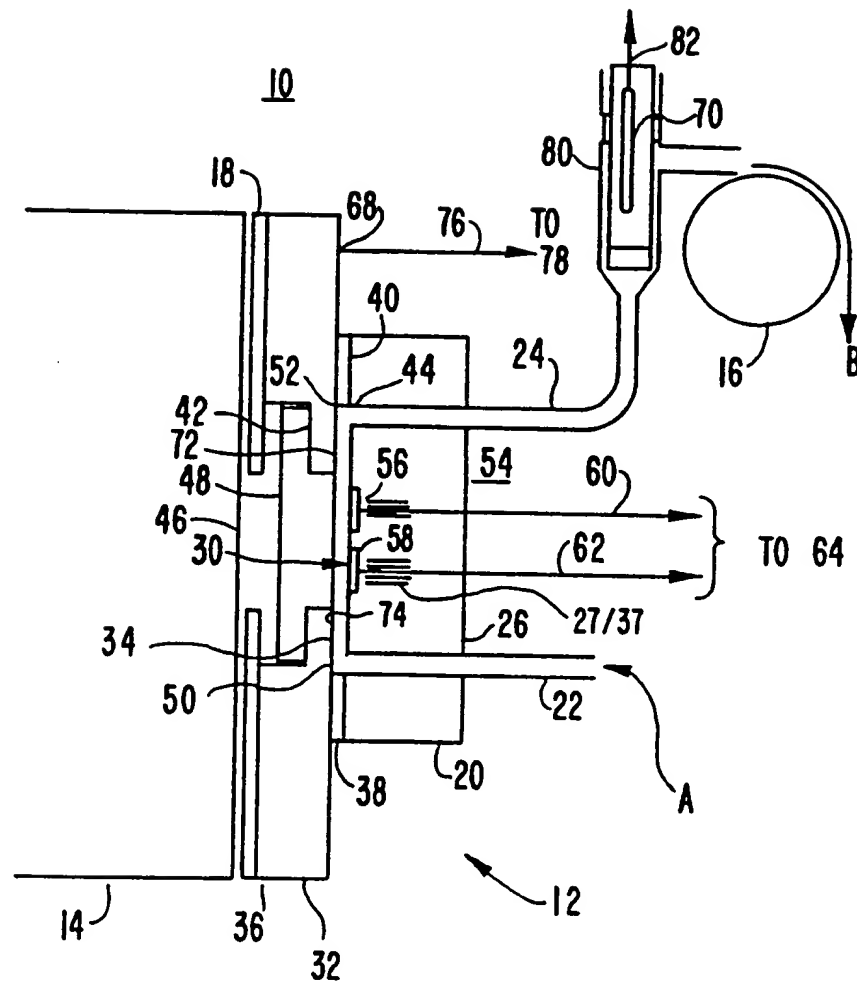
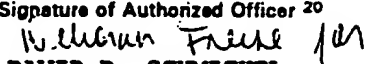


FIG. 25

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00992

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): G01N 21/66 US CL : 422/52		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
U.S.	422/52, 56; 435/7.1; 436/526	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>5</sup>		
DIALOG, BIOSIS, WPI, APS SEARCH ITEMS: ECL, LUMINESCENCE, BINDING, MAGNETIC		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category <sup>6</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y/X	US, A 4,628,037 (CHAGNON ET AL) 09 DECEMBER 1986. SEE ENTIRE DOCUMENT.	29-45/47-49
Y/X	CLIN. CHEM. VOLUME 37, NO. 9, ISSUED SEPTEMBER 1991, J.H. KENTON ET AL, "RAPID ELECTROCHEMILUMINESCENCE ASSAYS OF POLYMERASE CHAIN REACTION PRODUCTS", PAGES 1626-1632. SEE ENTIRE DOCUMENT.	1-49/50-53
Y/X	BIOCHEM. BIOPHYS. RES. COMM. VOLUME 128, NO.2, ISSUED 30 APRIL, 1985, Y. IKARIYAMA ET AL, "ELECTROCHEMICAL LUMINESCENCE-BASED HOMOGENEOUS IMMUNOASSAY", PAGES 987-992. SEE ENTIRE DOCUMENT.	4-49/1-3
A	NUCLEIC ACIDS RESEARCH VOL. 16, NO.11, ISSUED 1988, M. S. URDEA ET AL., "A COMPARISON OF NON-RADIOISOTOPIC HYBRIDIZATION ASSAY METHODS USING CHEMILUMINESCENT AND ENZYME LABELED SYNTHETIC OLIGODEOXYRIBONUCLEOTIDE PROBES", PAGES 4937-4956.	1-49
Y	J. ALLERGY AND CLIN. IMM. VOLUME 61, NO. 1, ISSUED JANUARY 1978, J. L. GUESDON ET AL, "MAGNETIC ENZYME IMMUNOASSAY FOR MEASURING HUMAN IGE", PAGES 23-27. SEE ENTIRE DOCUMENT.	29-45
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>15</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>2</sup>	Date of Mailing of this International Search Report <sup>2</sup>	
04 MAY 1992	20 MAY 1992	
International Searching Authority <sup>1</sup>	Signature of Authorized Officer <sup>20</sup>	
ISA/US	 DAVID B. SCHMICKEL	

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	CLIN. CHEM. VOLUME 26, NO. 6, ISSUED 1980, M. POURFARZANEH ET AL., "CORTISOL DIRECTLY DETERMINED IN SERUM BY FLUOROIMMUNOASSAY WITH MAGNETIZABLE SOLID PHASSE", PAGES 730-733. SEE ENTIRE DOCUMENT.	29-45
A	US, A, 4,652,533 (Jolley) 24 MARCH 1987. SEE ENTIRE DOCUMENT.	1-49
A	WO, A, 86/05815 (HILL ET AL) 09 OCTOBER 1986. SEE ENTIRE DOCUMENT.	1-45
A	WO, A, 87/06706 (MASSEY ET AL) 05 NOVEMBER 1987. SEE ENTIRE DOCUMENT.	1-53

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter (1) not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:
  
3. ☐ Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 1-49, drawn to a binding assay and an apparatus to perform the assay, classified in class 422 subclass 52.
- II. claims 50-53, drawn to a composition to perform assay and a kit containing it classified class 422, subclass 61.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. (Telephone Practice)
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
  
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
  
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y,P	US, A, 5,068,088 (HALL ET AL) 26 NOVEMBER 1991. SEE THE ENTIRE DOCUMENT.	46-49
Y,T	US, A, 5,093,268 (LEVENTIS ET AL) 03 MARCH 1992. SEE ENTIRE DOCUMENT.	46-49
Y,P	US, A, 5,061,445 (ZOSKI ET AL) 29 OCTOBER 1991. SEE ENTIRE DOCUMENT.	46-49
Y	US, A, 4,431,919 (KOSTLIN ET AL) 14 FEBURARY 1984. SEE ENTIRE DOCUMENT.	46-49
Y	CLIN. CHEM. VOLUME 26, NO.9, ISSUED SEPTEMBER 1980, RS KAMEL ET AL, "MAGNETISABLE SOLID PHASE FLUOROIMMUNOASSAY OF PHENYTOININ IN DISPOSABLE TEST TUBES" PAGES 1281-1284. SEE ENTIRE DOCUMENT.	29-45
A	EP, A, 0, 030 087 (SMITH) 06 OCTOBER 1981. SEE THE ENTIRE DOCUMENT.	1-53



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